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Annual Meeting Abstracts

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CONTINUING MEDICAL EDUCATION (CME)

This activity ("Association for Molecular Pathology 2011 Annual Meeting") has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the Federation of American Societies for Experimental Biology (FASEB), the American Society for Investigative Pathology (ASIP) and the Association for Molecular Pathology (AMP). FASEB is accredited by the ACCME to provide continuing medical education for physicians.

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GENETICS: G42, G51, G60, G64
HEMATOPATHOLOGY: H02, H04, H18, H20
INFECTIOUS DISEASES: ID11, ID45, ID50, ID65
SOLID TUMORS: ST11, ST14, ST24, ST56

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Abstract	Author	Relationship
ID45	Carolyn M. Hines	Employment – Glenview Terrace
ID45	Lance Peterson	Consulting – Cepheid, GeneOhm, GSK, MicroPhage, Nanogen, Roche, 3M, Wyeth (Pfizer) Research grants – Bayer, Cepheid, BD GeneOhm, GSK, Johnson & Johnson, Merck, MicroPhage, Nanogen, Nanosphere, Roche
ST11	Jack X. Yu	Employment – Ortho Clinical Diagnostics (a Johnson & Johnson company)
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ST11	John Jiang	Employment – Ortho Clinical Diagnostics (a Johnson & Johnson company)
ST11	Yi Zhang	Employment – Ortho Clinical Diagnostics (a Johnson & Johnson company)
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ST14	Sherman Chang	Employment and Stock Options – Derm Tech International
ST14	Tara Palmer	Employment and Stock Options – Derm Tech International
ST14	William Wachsman	Non-compensated scientific advisor – Derm Tech International

GENETICS

G01. An Uncommon Prothrombin Gene Variant in an African-American Woman with a Stroke

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Introduction: Mutations in coagulation Factor II (Prothrombin) and Factor V are the most prevalent hereditary risk factors for thrombophilia. Prothrombin mutation 20210 was first described in 1996; it consists of a G to A mutation that leads to increased prothrombin levels in blood. The mutation is found in up to 2% of the Caucasian population but is rare in people of African or Asian descent. Carriers and homozygous individuals are at increased risk of developing deep vein thrombosis as well as obstetric and fetal complications. In this study we describe the identification of an uncommon prothrombin variant in a patient who presented with a stroke. **Methods:** A 47-year-old black female from Jamaica with a history of hyperlipidemia and syncope was admitted to the stroke service with right sided hemiplegia (leg weaker than arm) and expressive aphasia. The MRI revealed a left frontoparietal infarct. The TEE showed evidence of a patent foramen ovale. Genomic DNA was extracted from peripheral blood and analyzed by real-time PCR for Factor V Leiden and prothrombin mutations. **Results:** The prothrombin 20210 assay showed an abnormal melting curve that was not consistent with wild-type or the 20210 G>A mutation. The melting curve showed two peaks, one at 59°C (wild-type allele) and an unknown peak at 53°C (the 20210 G>A mutation gives a peak at 49°C). To further characterize this unusual finding this region of the prothrombin gene was sequenced. The patient's DNA was amplified with primers PT5' and PT3" (PT5': 5'-TCTAGAAACAGTTGCCTGGC-3' and PT3': 5'-ATAGCACTGGGA-GCATTGAAGC-3'). The amplification product was then purified and sequenced by the Sanger method using fluorescent labeled dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. The sequence showed the presence of a C to T transition at position 20209; the normal G was present at position 20210. The Factor V Leiden mutation was not present. **Conclusions:** This patient is heterozygous for a 20209 C>T mutation. This rare mutation has been previously described in a small number of Middle Eastern and African-American patients in whom it was associated with recurrent spontaneous abortions, intrauterine growth retardation or neonatal demise. In this patient the 20209 C>T mutation was associated with stroke rather than obstetrical complications. This case also illustrates how real-time PCR coupled with melting point analysis can detect new or unusual variants that may be missed by other molecular techniques.

G02. Laboratory Validation of the Abbott Cystic Fibrosis Genotyping Assay on the Applied Biosystems 3500 xL Instrument

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Introduction: Cystic fibrosis (CF) is one of the most common inherited disorders. It is a complex multisystem disorder but the majority of disease morbidity and mortality is associated with pulmonary manifestations. Mutations within the *CFTR* gene have been shown to be responsible for this autosomal recessive disease. The Abbott Cystic Fibrosis Genotyping Assay is a robust assay that is FDA cleared for IVD use on the Applied Biosystems 3100 instrument. It detects 32 mutations as part of a core panel and additional polymorphisms in a separate panel. The Applied Biosystems 3500 xL is the newest generation capillary electrophoresis instrument and offers significant performance advantages over the 3100 instrument. **Methods:** A panel of samples containing all 23 ACMG recommended mutations was obtained from Coriell along with 10 samples of known *CFTR* intron 8 poly-T genotype, 27 laboratory confirmed *CFTR* negative samples, as well as the SeraCare Accurun 632 CF control. The samples were amplified and the OLA performed according to the IVD protocol in an Applied Biosystems 9700 thermocycler. Following amplification and OLA, the samples were subjected to capillary electrophoresis and analysis on an Applied Biosystems 3100 and an Applied Biosystems 3500 xL with both utilizing POP-6 polymer. The amplification, OLA, capillary electrophoresis, and analysis of all study samples were performed multiple times on multiple days to thoroughly evaluate the performance of the 3500 xL instrument for the CF assay. **Results:** In all cases, the identical *CFTR* and intron 8 poly-T genotype was obtained by both the 3100 and 3500 xL instruments. However, the 3500 xL offered several operator convenience and assay performance advantages versus the 3100. The pre-formulated instrument consumables such as the polymer pouch, cathode and anode buffer containers, and capillary array provided an ease of use advantage and the integrated RFID tags on these products allowed improved instrument QA and QC. Additionally, the 3500 xL offered increased sample throughput due to decreased electrophoresis time and increased sample capacity. All of these instrument and software improvements offer significant advantages when running the Abbott CF Genotyping Assay on the 3500 xL. **Conclusions:** The Abbott CF Genotyping Assay produces identical genotyping results on the Applied Biosystems 3500 instrument when compared to the 3100. Given its robust and reliable performance during this validation study and the significant advantages of the 3500 xL, it can be validated as a viable instrument for the Abbott CF Genotyping Assay in the clinical laboratory.

G03. Comparison of the Performance of Micofludics with Capillary Electrophoresis as a Platform for PCR Analysis for *FMR1* Premutations

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Introduction: Usage of capillary electrophoresis for the evaluation of PCR products to assess the expansions of the CGG triplet repeats in the 5'UTR of the *FMR1* gene is becoming a standard approach for Fragile X testing. Direct amplification of the CGG repeat region with flanking primers for allele sizing and triplet repeat primed PCR (TRP-PCR) for the detection of expansions as "stutter bands" are among the most common PCR applications. In both these assays fluorescently labeled primers are utilized and the generated amplicons are visualized and detected by capillary electrophoresis. Our objective was to evaluate the performance of a commercially available microfluidics platform and compare it to capillary electrophoresis. **Methods:** Archived genomic DNA specimens (n=47), previously tested for *FMR1* expansions were analyzed. A commercially available *FMR1* test kit (Abbott Diagnostics) was utilized. Our testing strategy involved using "primer 1" for allele sizing initially. If the results could not be ascertained with "primer 1", the TRP-PCR experiments were done using "primer 2". Electrophoresis of PCR products was performed on the 3130XL Genetic Analyzer (Applied Biosystems) and 2100 Bioanalyzer (Agilent Technologies). **Results:** Forty-seven DNA samples were tested using "Primer 1". 32 samples had normal alleles, 4 samples had premutations (80-144 repeats) and 11 female samples showed single bands after capillary electrophoresis. The analysis of the PCR products on microfluids revealed identical results. The eleven samples with single peaks were also analyzed by TRP-PCR using "Primer 2", *FMR1* expansions were detected in three cases. **Conclusions:** Long run time (~4hrs) and the high cost of fluorescent primers are the issues with capillary electrophoresis for assessment of *FMR1* expansions. The advantage of a microfluidics based platform is shorter run time (30 minutes) and possibility of using non-florescent primers. Our experiments established the proof of principle that the microfluidics based electrophoresis can be utilized for *FMR1* testing.

G04. Long-Range PCR for Clinical Mutation Detection of *SFTPA1* and *SFTPA2* Genes

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Introduction: Germ line mutations in Surfactant Protein A (SP-A) are correlated with severe pulmonary disease. SP-A exists as two isoforms, SPA-1 (*SFTPA1*) and SPA-2 (*SFTPA2*); these surfactants serve two main roles in the lungs: alveolar gas exchange and innate immune defense. Both genes are located on the q arm of chromosome 10 and have >98% and >90% sequence identity in their coding and non-coding regions, respectively. The high identity of the two genes makes traditional PCR design and DNA sequencing exceptionally challenging. We have developed a genetic DNA sequencing test based on long-range PCR for the clinical detection of mutations in *SFTPA1* and *SFTPA2*. **Methods:** Primer pairs were designed to specifically amplify approximately 10 kb of genomic sequence for both *SFTPA1* and *SFTPA2*. PCR amplification using Takara 'One Shot LA PCR' was performed for each gene on genomic DNA extracted from whole blood. The two PCR products were purified using Qiagen's 'QIAquick PCR Purification microcentrifuge' kit. Each PCR product underwent restriction enzyme digestion with EcoRV to confirm the specific identity of *SFTPA1* or *SFTPA2*. The PCR products were sequenced using internal primers with Applied Biosystems (AB) BigDye Terminator 3.1 and purified using AB BigDye Xterminator kit. Capillary electrophoresis of the sequenced fragments was performed on an AB 3130xl to generate chromatograms. The chromatograms were analyzed and interpreted using AB Sequence Analysis, FinchTV (Geospiza), Mutation Surveyor (Softgenetics), and Alamut (Interactive Biosoftware). **Results:** Gel electrophoresis demonstrated the distinct banding patterns that are specific for *SFTPA1* and *SFTPA2* PCR products digested by EcoRV. Bi-directional DNA sequence was obtained for all coding sequence with quality scores in Mutation Surveyor greater than 20.The chromatograms showed gene-specific alleles at areas of non-homology for all four exons in the two genes. Samples with previously reported mutations were accurately called by the mutation software. **Conclusions:** Although long-range PCR is not typically used for clinical sequencing and mutation detection, it is useful for genes such as *SFTPA1* and *SFTPA2*, which have high similarity and limited unique sequences for priming. In the research setting, investigators of these genes have been limited to priming sites that also contain single-nucleotide polymorphisms of unknown significance. Using long-range PCR, we are able to avoid priming sites that contain single-nucleotide polymorphisms and specifically

amplify *SFTPA1* and *SFTPA2* in individual reactions. As additional clinically significant genes are discovered that have isoforms possessing high sequence identity, long-range PCR may have a more prominent role in clinical DNA sequencing tests.

G05. Homocysteine and *MTHFR* Gene Polymorphism in Alzheimer's Disease and Other Dementia

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Introduction: The elderly population rise due to increased life expectancy has also resulted in an increased prevalence of dementia. Alzheimer's disease (AD) is characterized by progressive cognitive decline and is the most common type of late-life dementia. However, the pathogenesis of AD has not been fully understood. Genetics and vascular components such as atherosclerosis play a role in the onset of the disease. This study aimed to analyze relationships between *MTHFR* gene polymorphism and homocysteine, lipid profiles, folate, and vitamin B12 in dementia including Alzheimer's disease and minor cognitive impairment. **Methods:** A total of 151 patients with complaints of memory decline were included in this study. Patients were divided into four groups, AD (N=26), vascular dementia (N=7), minor cognitive impairment (N=27) and normal cognitive function (control group, N=91) according to MMSE scores and clinical criteria by NINCDSADRDA. *MTHFR* gene polymorphisms were determined using Seeplex *MTHFR* C677T/A1298C kit (Seegene, Seoul, Korea). Homocysteine levels were tested using Hitachi 7600 (Hitachi High-technology corporation, Tokyo, Japan). Folate and vitamin B12 levels were determined using Advia Centaur (Simens Healthcare Diagnostic, NY, USA). We also determined e4 allele of apolipoprotein E gene (APOE). **Results:** Homocysteine, folate, and vitamin B12 were not significantly different among the four groups. However, homocysteine levels were significantly increased in AD (13.87 μEq/L) compared to the control group (7.66 μEq/L)P=0.007). Our data showed no significant difference in frequencies of *MTHFR* 677 and 1298 genotypes among the four groups. Divided into six groups according to haplotypes of *MTHFR* 677 and 1298, homocysteine levels did not significantly differ among these groups. However, homocysteine levels were significantly increased in 677TT compared to 677CC (P=0.015). Apolipoprotein E genotyping: e2e3 (8.7%), e2e4 (1.4%), e3e3 (65.2%), e3e4 (24.6%). The frequencies of the APOE genotypes did not differ significantly among the four groups (P = 0.350). APOE e4 allelic frequencies were 43.8% in the AD group and 23.5% in the control group. APOE e4 carriers had significantly decreased HDL-C and apoA1 levels as compared to APOE e4 non-carriers (P < 0.001 and P = 0.001, respectively). **Conclusions:** Homocysteine levels were significantly increased in AD and in *MTHFR* 677TT genotype. Longitudinal studies including a larger patient cohort are needed to confirm these results and evaluate vitamin therapy for AD or minor cognitive impairment.

G06. Assessment of a Novel Point-of-Care Assay to Detect the *CYP2C19*2* Single Nucleotide Polymorphism

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Introduction: Data suggest that the *CYP2C19*2* variant is an important determinant of a patient's pharmacologic and clinical response to clopidogrel, and this information has been incorporated into the updated FDA label. Accordingly, molecular laboratories may be requested with increasing frequency to rapidly assess for this variant to aid clinicians in selection and/or dosing of antiplatelet therapy. As part of the ELEVATE-TIMI 56 clinical trial, we compared a novel point-of-care assay for *CYP2C19*2* to a sequencing-based assay. **Methods:** A Research Use Only (RUO) prototype of the Nanosphere Verigene 2C19/CBS Nucleic Acid Assay (which assesses *2-*10, *13 and *17) and a Pyrosequencing assay (laboratory-developed) were used to assess *CYP2C19*2* status in peripheral blood genomic DNA from 333 ELEVATE-TIMI 56 patients; samples were individually tested by the Nanosphere assay using whole blood and were batch-tested by Pyrosequencing after DNA isolation and PCR. We compared the concordance rate, turnaround times (TAT), hands-on times (HT) and other features. **Results:** Genotype results confirmed by both methods were: wild type (at *2 locus) 245/333 (74%); heterozygous *2 allele 80/333 (24%); homozygous *2 allele 6/333 (1.8%). The concordance rate between the two methods was >99% (331/333). The two cases with initially discrepant results (heterozygotes by Verigene, wild type by Pyrosequencing) were repeated and determined to be wild type by both methods. TAT for Verigene was ~3.5 hours with 15 minutes HT per individual sample. Pyrosequencing (including DNA isolation and PCR) had a TAT of ~5 hours with a HT of 90-120 minutes per batch. Verigene was relatively simple to operate and assessed 10 additional CYP2C19 SNPs whereas Pyrosequencing was designed to only assess *CYP2C19*2*. The Pyrosequencing platform, due to the 96-well plate format, had a greater capacity to simultaneously run larger batches of specimens. **Conclusions:** Both the RUO prototype of the Nanosphere Verigene 2C19/CBS Nucleic Acid Assay and laboratory-developed Pyrosequencing were robust, accurate platforms for assessment of *CYP2C19*2*. Pyrosequencing was technically more complex and had a slower turnaround time, but appears well suited for laboratories with high daily testing volumes that choose to batch-test specimens and assess fewer SNPs. The Verigene assay with its simple operation and multiplexing capability appears well suited for either point-of-care operation or for laboratories with moderate daily testing volumes that require faster turn around and prefer to test specimens individually.

G07. Rapid Targeted Prenatal Detection of Sex Chromosomal Aneuploidies Using Quantitative Fluorescence Polymerase Chain Reaction in Singapore

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Introduction: Conventional cytogenetic analysis for prenatal diagnosis requires 7-14 days before results are released. This long waiting time can cause parental anxiety. Common fetal chromosomal aneuploidies can be detected by rapid targeted tests such as interphase fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA) and quantitative fluorescent PCR (QF-PCR). We have developed QF-PCR as a clinical test to detect fetal autosomal aneuploidies. To detect sex chromosome aneuploidies, we now explore additional microsatellite markers located on chromosomes X and Y to be included into our established QF-PCR test. **Methods:** A total of eight markers [polymorphic (*DXS6785*, *DXS6789*, *DXS6803*, *DXS6809*, *XHPRT*, *X22*); non-polymorphic (*AMXY*); chromosome Y-specific (*SRY*)] were analyzed. Assay validation was performed with 24 archived DNA samples with known sex chromosome abnormalities determined by cytogenetic analysis [45,XO(n=7), 47,XXX(n=2), 47,XXY(n=8), 47,XYY(n=1), mos45,XO/46,XY(n=2), mos45,XO/46,XX(n=1), mos45,XO/46,X,r(X)(p22.3q28)(n=1), 46,X,psu idic(X)(p11.1)(n=1), 46,X,i(X)(q10)(n=1)]. In our blinded study, DNA was isolated from 2 ml of amniotic fluid collected from mothers undergoing routine amniocentesis at 18-22 gestational weeks (n=100). **Results:** In our validation study, 45,XO, mos45,XO/46,X,r(X)(p22.3q28) and mos45,XO/46,XY samples showed single allele in each X-chromosome marker, with the presence of Y-chromosome-specific alleles (*SRY, AMY*) in mos45,XO/46,XY samples. mos45,XO/46,XX showed two alleles in all X-chromosome markers and was unable to detect monosomy X. Both 46,X,psu idic(X)(p11.1) and 46,X,i(X)(q10) samples showed the presence of an additional X-chromosome. XXY and XYY samples were detected with double allele dosage of X- or Y-chromosome markers with the addition of single allele dosage of Y- or X-chromosome markers respectively. In our blinded study, all samples (n=100) showed normal male and female chromosomes (XY,n=47; XX,n=53) as confirmed by their respective karyotypes (100 percent specificity, lower 95 percent CI, 95.4). **Conclusions:** QF-PCR can detect monosomy X and mosaic monosomy X/XY at 23.8-50 percent mosaicism, and unable to detect mosaic monosomy X/XX. The presence of an additional dosage of Xq markers does not necessary indicate trisomy X, as shown in psu idic(X)(p11.2) and i(Xq) samples with Xq duplication. Additional Xp markers are required. Our present panel of markers can detect XXY, XYY, normal XX and XY with 100 percent specificity. Karyotyping is recommended in the presence of abnormal results. Although QF-PCR is unable to detect abnormalities in chromosomal structure such as ring chromosome and balance translocation, sex chromosome aneuploidies can be detected. Results can be released within 48 hours of sample collection, alleviating parental anxiety and improving clinical management of affected pregnancies.

G08. Sanger Sequencing Assay to Detect 2C19 Allele Variants Associated with Plavix Metabolism

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Introduction: Plavix is given to patients with cardiovascular disease in order to reduce the risk of heart attack, unstable angina, stroke, and cardiovascular death. The drug is metabolized to its active form primarily by the *CYP450* isoenzyme 2C19. The *CYP450* super-family of genes displays varying degrees of intra-family homology; specifically 2C19 has high homology with 2C9, which generates inherent challenges for complex detection assays. Here we have designed a Sanger sequencing based assay to detect those 2C19 alleles with clinical associations. **Methods:** PCR and sequencing primers were developed and used to amplify 2C19 gene 5' UTR and exons 1, 3, 4, 5 and 8. Sanger sequencing assays were performed on DNA from the Coriell repository and CAP proficiency surveys using BigDye Terminator Mix version 3.1 (ABI). Sequence analysis was done on a 3130 genetic analyzer using sequencing software version 5.3.1 (ABI). 2C9 sequence-exclusions were confirmed by interrogating 2C19 non-homologous regions. **Results:** 2C19 sequences were generated for each of the target exons and 5' UTR. These sequences were compared to NCBI reference data to detect

*2 681G>A, *3 636G>A, *4 1A>G, *8 358T>C, *9 431G>A *10 680C>T, *13 128C>T and *17 -806C>T alleles. All genotypes generated by our Sanger sequencing assay were 100% concordant with those known genotypes used in the analyses. **Conclusions:** Stringent primer design allowed *CYP2C19* alleles to be amplified in the absence of contaminating 2C9 sequences. This allows more accurate elucidation of *CYP2C19* genotypes and predicts a more accurate metabolizer phenotype.

G09. Novel *FLCN* c.1490_1491delTG Mutation in a Patient with Spontaneous Pneumothorax

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Introduction: The cause of primary spontaneous pneumothorax (PSP) is obvious. Recently, the *FLCN* mutation was suggested to be a causal factor in PSP. Herein, we report a novel *FLCN* mutation in a patient with PSP. **Methods:** A 43-year-old Korean female patient with chief complaint of repetitive PSP had numerous emphysematous bullae and multiple large cysts based upon high resolution computer tomography. Genomic DNA and total RNA were isolated from venous blood, and cDNA was synthesized. All coding exons and flanking intronic regions of *FLCN* gene were amplified by PCR and directly sequenced. Also, we amplified and sequenced the entire coding region of *FLCN* mRNA. For determining the amount of *FLCN* transcripts of the patient compared with normal persons (n=20), we performed the relative quantitative real-time ddCt analysis compared with ABL transcripts. All relative quantitative real-time RT-PCR were executed in triplicate. **Results:** The *FLCN* c.1490_1491delTG (p.Val497Glyfs*22) mutation in exon 13 was found in the genomic DNA and mRNA levels. Quantitative real-time RT-PCR revealed a similar amount ($2^{-\Delta\Delta Ct}$ = 0.99) of *FLCN* transcripts from the PSP patient compared with normal persons. This mutation can escape nonsense-decay system, because premature termination codon generated by c.1490_1491delTG mutation is located less than 50-55 bp downstream of the last exon 13 to exon 14 junction. **Conclusions:** We identified a novel *FLCN* c.1490_1491delTG mutation in a patient with PSP.

G11. Validation of INFINITI Microarray System for Pharmacogenetic Testing of *CYP2D6* for Tamoxifen Therapy

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Introduction: Tamoxifen is an estrogen receptor (ER) antagonist that blocks estrogen binding to ER leading to the inhibition of ER+ cancer cells. The pro-drug tamoxifen is converted into active metabolite endoxifen by Cytochrome P450 (CYP) enzymes, primarily by *CYP2D6*. Formation of active metabolite is associated with the variants of *CYP2D6* gene. Loss of function mutation in *CYP2D6* can impair the metabolism of tamoxifen. Consequently, ER+ breast cancer patients with the loss of function variants in *CYP2D6* may not benefit from tamoxifen therapy. Identifying these patients by genotyping *CYP2D6* gene variants will be useful to tailor individualized breast cancer therapy. **Methods:** Genomic DNA isolated from 47 split-specimens that were previously genotyped for *CYP2D6* and 16 Coriell DNAs of known genotypes were analytically validated by genotyping with a FDA cleared commercial platform INFINITI from AutoGenomics. The INFINITI employs an array-based assay (CYP2D6I) encompassing 15 variants. The assay involves a multiplex PCR amplification of genomic DNA followed by allele-specific primer extension using fluorescently labeled dCTP and hybridization on to a micro-array coated with capturing oligonucleotides specific for the primer-extended products. A built-in confocal microscope is enabled to capture fluorescent signal from the pre-determined hybridization spots corresponding to specific alleles and genotypes deciphered from the signal ratio. For analytical validation, we have determined accuracy, method comparison, intra- and inter-assay precision, sensitivity and specificity of the CYP2D6I assay. **Results:** Accuracy of the CYP2D6I assay, determined by comparing the consistency in genotyping calls between known and observed results, was in total agreement for fourteen alleles common to both methods for split specimens and the known variants of Coriell DNAs. Three specimens genotyped in six replicates within the same run and in duplicates over five days yielded reproducible results without any discrepancy. Sensitivity of the assay determined with three specimens in duplicates demonstrated that accurate genotype calls could be generated with DNA amount ranging from ~5 ng to 150 ng per reaction compared to the recommended level of 75 ng DNA per reaction. The assay was specific as there was no false positive or false negative, the template-free control remained negative and the hybridization and background controls met the assay criteria. **Conclusions:** *CYP2D6* assay using the INFINITI platform has been analytically validated with satisfactory results. Performance for accuracy, precision, sensitivity and specificity has shown that *CYP2D6* assay is reliable. Although this assay can genotype fifteen variants, clinical utility of many of the ethnocentric variants are yet to be established.

G12. Detection and Characterization of Fragile X Expansion Mutations Using PCR-based Assays

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Introduction: Fragile X diagnostic testing involves determining the size of the CGG repeat in the *FMR1* promoter and the level of promoter methylation. PCR amplification of the *FMR1* CGG repeat in combination with Southern Blot (SB), despite being laborious and time-consuming, is commonly used for this purpose. In this research study we evaluated whether three laboratory-developed (LD) PCR-based protocols could reliably identify, size, and determine promoter methylation status of expanded *FMR1* alleles. **Methods:** Ninety-one clinically derived DNA samples (previously analyzed and blinded by RUMC) containing *FMR1* full mutations, premutations, and mosaic mutations were tested by the LD *FMR1* screening and sizing protocols. A subset (n=20) was assessed for methylation at the *FMR1* promoter using quantitative real-time PCR. Briefly, genomic DNA samples were bisulfite converted using a commercial kit. Novel oligonucleotides to ActinB (ACTB) enabled us to control for the amount of input DNA that was successfully bisulfite converted and pipetted. Novel oligonucleotides to the *FMR1* promoter region spanning several CpGs, were used to amplify DNA that was methylated and had been bisulfite converted. Our results were compared to results from in-house combined PCR-Southern blot methodology obtained at RUMC. **Results:** Fifty-two samples were identified as expanded by the screening protocol and the remaining 39 were determined to contain normal *FMR1* alleles. We found 100% concordance in identifying premutation, full mutation and mosaic samples. Among the samples tested, 18 had expansions only within the premutation range (55-200 repeats), 19 had expansions only within the full mutation range (>200 repeats, with 17 having large expansions of >1000 repeats), and 15 had distinct expansions both within the premutation and full mutation range (pre-/full mutation mosaics). Among 20 samples tested using the novel *FMR1* methylation assay, we detected 0% methylation in three normal males (m), 42% to 52% in four normal females (f), 6% to 57% in three premutation samples (m+f), 33% to 99% in seven mosaic samples (m+f) and 85-100% in three full mutation males. Eighteen of 20 results were in good agreement with the Southern blot data. Two samples were quantitatively different (87% vs 100% SB and 72% vs 40% SB), possibly reflecting different CpG targets tested by the two methods. **Conclusions:** These lab-developed protocols are rapid and reliable methods for the detection and characterization of expanded FX alleles. PCR-based assays are desirable for laboratories conducting FX clinical testing as they quickly identify the presence of expansion mutations; determine allele size, and quantify the level of *FMR1* promoter methylation.

G13. *HLA-B*5801*, a Strong Pharmacogenetic Marker for Severe Cutaneous Adverse Drug Reaction Caused by Allopurinol in Thai Population

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Introduction: Allopurinol is the most common drug used for lowering uric acid and for treatments of hyperuricemia and gouty arthritis. This drug has been reported as a common culprit drug for severe cutaneous adverse drug reactions (SCADR) including Stevens–Johnson syndrome (SJS), Toxic epidermal necrolysis (TEN) and Hypersensitivity syndrome (HSS) in several countries. Although a strong association between allopurinol-induced SCADR and *HLA-B*5801* has been reported in Han Chinese, only a moderate association was observed in other populations. The present study aimed to investigate the association between allopurinol-induced SCADR and *HLA-B*5801* in a Thai population. **Methods:** Forty-one allopurinol-induced SCADR and 81 allopurinol-tolerant patients were recruited in the study. Genomic DNA was extracted from peripheral blood. The presence of *HLA-B*5801* allele in these patients was determined using a PG5801 DNA detection kit. **Results:** The *HLA-B*5801* allele was detected in all of the allopurinol-induced SCADR patients (41/41, 100%) but only 10 out of 81 allopurinol-tolerant patients (12.40%). The risk of allopurinol-induced SCADR was markedly higher in patients with *HLA-B*5801* when compared with those that did not carry this allele. The sensitivity and specificity of the *HLA-B*5801* allele for prediction of allopurinol-induced SCADR were 100% and 87.65%, respectively. **Conclusions:** The *HLA-B*5801* is a good pharmacogenetic marker for screening of Thai patients who are at higher risk of allopurinol-induced SCADR.

G14. Association of Chromosome 9p21 Polymorphism with Coronary Artery Disease in Western Indians

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Introduction: Indians have a high propensity to develop Coronary Artery Disease (CAD) and it has been estimated that 60% of the world's cardiac population will be in India by the year 2020. In this context it is very important to evaluate the new markers especially genetic variants in the Indian population, which is a heterogenous group, in addition to the traditional risk factors. Genome-wide studies have implicated the 58kb region of chromosome 9p21 to be associated with CAD. Several SNPs were implicated in this region (viz rs1333049, rs10757278, rs10757274, rs2383206, rs1004638 rs10116277, rs1333040, rs2383207, rs1994016 etc). In the current study we have evaluated the association of SNP rs10757278 A/G at the 9p21 locus with CAD, especially in Western Indians (Indo-European population). **Methods:** Genotyping for rs10757278 A/G was done by direct sequencing in 215 cases with confirmed CAD and 150 controls. To determine association of the variant rs10757278 with CAD, both univariate and multivariate analysis were performed. For the calculation of the Framingham Risk Score (FRS) with the 9p21 information a value of 1, 0, and -1 was given for the genotype GG, AG, AA, above that determined by the traditional FRS score and the number of individuals getting reclassified determined. **Results:** Statistically significant differences were seen in the smoking status (P<0.01), presence of family history (P<0.0001), diabetes (P<0.0001) as well as hypertension (P<0.0001) in cases vs controls. The reported minor allele frequency (0.53 in controls) for this SNP in our population is almost the same as in other ethnic groups except African Americans. A higher frequency of the rs10757278 G allele was seen in cases as compared to the controls (0.64 vs 0.53), and associates with increased risk for CAD (OR 1.832 per G allele 95% 1.035-3.242, P 0.042) in the univariate analysis. In the stepwise backward logistic regression model LDL levels (P<0.0001) and 9p21 rs10757278 variant (P=0.035) were associated with the risk of CAD. No association with prevalent myocardial infarction as the event versus CAD, was seen in a univariate analysis. Addition of the 9p21 allele to Framingham risk score FRS, resulted in a shift of 17% of individuals from the low risk category to the intermediate-low (>5% to <10% 10-year risk) and 7% from intermediate-low to intermediate-high (>10% to <20% 10-year risk) categories. **Conclusions:** In conclusion the 9p21 locus is significantly associated with the risk of CAD in the select Indian population.

G15. *De Novo* Marker Chromosome Unraveled to be Chromosome 18 in a Girl with Mental and Motor Retardation

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Introduction: Conventional cytogenetic analysis is not sensitive enough to detect submicroscopic chromosomal rearrangements that lead to copy-number gains or losses that might be responsible for clinical phenotypes. Recently a new array based technology called array Comparative Genomic Hybridization (aCGH) has been used to detect submicroscopic chromosomal abnormalities that cannot be detected by conventional cytogenetic analysis. Using this technology allows us to detect submicroscopic genomic changes in children with idiopathic intellectual disabilities, mental retardation, autism, developmental delay and multiple congenital anomalies. **Methods:** In the present study, we analyzed a 6-year-old girl with mental and motor retardation. The parents requested genetic testing for their child and they also wanted to learn prenatal diagnosis opportunities for future pregnancies. Conventional cytogenetic analysis was performed and the karyotype was reported as 47,XX,+mar while the parents' karyotypes were 46,XX and 46,XY. For more detailed analysis, Roche NimbleGen Human CGH 3x720K Whole-Genome Tiling v3.0 Array was used. For the analysis of raw data web based Genoglyphix program (Signature Genomics) was used. **Results:** Five chromosomal regions were marked by the program automatically after analysis. Two of them were copy losses and three were copy gains. However, only one region was found to be clinically significant. This region was located at 18p11.32-18p11.21, and 1124 probes marked this region as a copy gain. This region comprises 72 genes and 43 of them are OMIM genes. Also copy gain in this region was found to be related to Holoprosencephaly 4 syndrome. The result was confirmed by fluorescent in situ hybridization (FISH) of metaphase spreads of the patient with whole chromosome probe 18, which hybridized with normal chromosomes 18 and the marker chromosome. **Conclusions:** Genome-wide array analysis gives us an ability to analyze submicroscopic chromosomal rearrangements and uncovered complex karyotypes that cannot be discerned by using conventional cytogenetic analysis. In the current case, after determining the marker chromosome, we were able to inform the parents about prenatal diagnostic procedures for future pregnancies.

G16. Large Deletions Resulting in Cystic Fibrosis in Three Patients and Discussion of the Methods Used

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Introduction: Cystic fibrosis (CF) is one of the most common autosomal recessive diseases for which mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene are responsible. Mutations in the *CFTR* gene include missense, nonsense, frameshift, splicing, and also small and large deletions and insertions. However, the frequency of the large genomic rearrangements such as large deletions or insertions are rare (2.57%). **Methods:** Here we present three patients who have large deletions in the *CFTR* gene with different diagnostic approaches. Genomic DNA was extracted from peripheral blood lymphocytes. *CFTR* gene mutations were analyzed by using reverse hybridization assay and a single multiplex semi-quantitative PCR (SMSQ-PCR) methods. **Results:** Case 1 is an 11-year-old girl who has a family history. Reverse hybridization assay revealed that the proband carried a homozygous R334W mutation. The father was heterozygous for R334W and the mother revealed normal genotype. Chromosome analyses were performed to exclude any chromosomal rearrangement involving the *CFTR* region. QF-PCR was performed and showed the different parental origins of chromosome 7 in the first child. Additionally, SMSQ-PCR was used to amplify the promoter and 27 exons of the *CFTR* gene. A deletion of exons 4 through 10 of the *CFTR* gene was found in a heterozygous state in the mother and in the proband, which explained the genotype and phenotype of the proband. There was no deletion or duplication detected in father's DNA. Case 2 is an 1-year-old girl, who was referred for *CFTR* mutation screening. As a result of the reverse hybridization assay, wild-type probes of intron 4 and 5, exon 4, 7, 9, 10 were not amplified and we thought that the patient had a large deletion including these regions. For confirmation of this result, reverse hybridization assay was also performed for the parents and results revealed normal genotype. Case 3 is a 1.5-year-old girl who had hyponatremia, metabolic alkalosis, persistent vomiting and malnutrition. It was found that the patient had a homozygous deletion in intron 12 by reverse hybridization. Parents did not accept mutation screening and other molecular studies for the confirmation of our results. **Conclusions:** Reverse hybridization assay is a useful and rapid method to analyze common mutations responsible for CF. However, in some rare cases such as having genomic rearrangements, results should be confirmed by another method like multiplex semi-quantitative PCR. Defining the molecular pathology is important for diagnosis and genetic counseling for future pregnancies.

G17. Molecular Genetic Testing for Fragile X Syndrome: Laboratory Performance on the College of American Pathologists Proficiency Surveys (2001-2009)

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Introduction: Molecular genetic testing for fragile X syndrome encompasses detection of the size of the CGG trinucleotide repeat region in the 5' untranslated region of the *FMR1* gene and of methylation associated with full mutations. The College of American Pathologists (CAP) offers biannual proficiency testing for molecular analysis of fragile X syndrome. The purpose of this study was to analyze laboratory performance on the CAP fragile X proficiency surveys from 2001 to 2009. **Methods:** Individual laboratory responses were analyzed for accuracy of genotype determination (normal, gray zone, premutation or full mutation) and size analysis of the *FMR1* trinucleotide repeat region. The sensitivity and specificity of testing for fragile X were calculated. The mean, median, and standard deviation of reported repeat sizes were calculated using Excel. Performance for repeat sizing was evaluated based on variance from the median values. **Results:** Overall, laboratories demonstrated an analytical sensitivity of 99% and 95.5% for detection of full mutations in males and females, respectively, a sensitivity of 98% for detection of premutations, and a specificity of 99.9%. Measurement of the size of the CGG repeat region demonstrated good interlaboratory concordance overall. An increase in the range of reported sizes was observed with increasing repeat number. For the majority of surveys, more than 90% of responding labs measured within two repeats of the median for normal alleles with 20-33 CGG repeats and within five repeats of the median for alleles with 42 repeats. For premutation samples with 90 repeats, an average of 86% of laboratories measured within ten repeats of the median result. For full mutation alleles, an average of 88% of laboratories reported results within two standard deviations from the median result. Performance for measuring repeat size in the premutation range improved over time, with a tighter distribution of sizes reported and a smaller percentage of labs reporting outlier results, coincident with an increased percentage of laboratories using capillary electrophoresis rather than gel-based PCR for fragment sizing. **Conclusions:** Molecular genetic testing for fragile X syndrome

demonstrated excellent sensitivity and specificity by laboratories participating in the CAP surveys. Allele sizing demonstrated good performance overall and has improved over the time frame analyzed for premutation alleles, where size analysis is important to determine risk of expansion to a full mutation in carrier females. Participation in proficiency testing is useful for laboratories to assess individual performance and to determine when they should recalibrate their assays.

G18. Performance of STR Genotyping in Samples Referred for Molar Pregnancy Testing

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Introduction: Molecular genotyping using PCR amplification of short tandem repeats (STRs) is an established method for accurately diagnosing and classifying complete and partial hydatidiform moles using DNA extracted from fresh or formalin-fixed, paraffin-embedded (FFPE) samples from products of conception (POCs). STR analysis can potentially identify other genomic imbalances, such as trisomy, which can also be implicated in early pregnancy losses and can morphologically mimic molar pregnancy on histologic examination. We reviewed all cases referred for hydatidiform mole testing by microsatellite genotyping in our institution to determine the technical performance of the STR assay and to investigate cases of potential trisomy. **Methods:** Genotyping was performed on 95 samples using a commercial multiplex PCR-based assay (Identifiler, Applied Biosystems), capillary electrophoresis (ABI 3100), and genotyping software (GeneMapper, Applied Biosystems). Biallelic peak area ratios were calculated by dividing the peak area of the longer allele (B) by the peak area of the shorter allele (A). Reference intervals for biallelic loci were established using maternal tissue (expected to be 1:1) and partial hydatidiform moles (expected to be 1:2 or 2:1), and these reference ranges were used to investigate possible trisomies in nonmolar pregnancies. A separate panel of STR primers for chromosomes 16, 18 and 21 was used to investigate nonmolar cases exhibiting possible trisomy, and these cases were also reviewed independently by two pathologists with expertise in placental pathology who were blinded to genotyping results. **Results:** Of the 95 samples tested, 41 were classified as molar pregnancies (24 complete and 17 partial moles). Among 53 nonmolar specimens, 11 had allelic ratios suggestive of trisomies (9 trisomy 16; 1 trisomy 18; 1 trisomy 21). One of the 95 samples could not be analyzed due to lack of amplifiable maternal DNA. Mini-STR primers confirmed trisomic allelic pattern in all of the cases with residual sample available for testing. Pathologic features were suggestive of molar pregnancy in all 11 cases with confirmed or suspected trisomy. One of the nonmolar specimens showed mosaicism for biparental and androgenetic cell lines, and this case was histologically classified as placental mesenchymal dysplasia on retrospective review. **Conclusions:** Trisomy can morphologically simulate pathologic features observed in hydatidiform moles. Microsatellite genotyping is valuable in the diagnosis of molar pregnancy and detect aneuploidy, which can be confirmed using additional STR markers on the suspected abnormal chromosome.

G19. Affymetrix 2.7Mb High Resolution Genome-Wide SNP Array in Clinical Practice

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Introduction: Pathogenic copy number variants (CNVs) are found in 5% to 15% of individuals with intellectual disability using different array platforms. It is expected that the arrays with higher resolution genome coverage detect CNVs more accurately and allow identification of smaller CNVs. **Methods:** We applied Affymetrix Cytogenetics Whole Genome 2.7M array to assess CNV detection in 20 cases (various microdeletion syndromes and cases with marker chromosome), which contain pathogenic CNVs previously detected. Also we applied array in 40 cases with intellectual disability or multiple congenital anomalies. **Results:** As a diagnostic test, detection rate of pathogenic CNV was 17.5% (7 in 40). Detected pathogenic CNVs included 0.9-Mb microdeletion at 2q22.3 (*ZEB* gene deletion: Mowat-Wilson syndrome) and 1.1-Mb microdeletion at 4q21.21q21.22 (novel microdeletion syndrome published in J. Med. Genet, 2010 47:377-84). One case showed two significant genomic imbalances that might originate from unbalanced translocations. One case showed 40-Mb long continuous stretches of homozygosity at 11q. Two cases had a small telomeric imbalance. However, confirmatory test with telomere MLPA could not detect any abnormalities. **Conclusions:** We validated Affymetrix microarray for the previously known microdeletion syndromes and concluded microarray analysis significantly improves the diagnostic yield and the quality of the diagnostic laboratory workflow. However, careful interpretation and confirmatory tests were needed in the case of small telomeric imbalances.

G20. Clinical Evaluation of Two Commercially Available PCR-Based Methods for the Detection of *FMR1* Mutation

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Introduction: The current workflow for diagnostic laboratories performing fragile X testing is time consuming and labor intensive. Additionally, current methods are limited in the range of repeat sizes and degree of mosaicism that can be detected. Recently-developed PCR-based assays simplify workflow, amplify full mutation alleles, and improve sensitivity for detecting low-level mosaicism. **Methods:** We evaluated the performance characteristics and workflow of two commercially available methods for determining *FMR1* mutation status using 36 known Coriell DNA samples and NIST standards and 40 previously tested anonymized patient samples. We tested 22 normal, 10 intermediate, 27 premutation, and 17 full mutation samples. We also tested each method's ability to detect mosaicism (ranges: 90% to 1% for males; 50% to 1% for females). One method was an RUO kit from Asuragen (AmplideX *FMR1* PCR reagents). Only the CGG primers were tested. The second method used a set of ASRs from Abbott Molecular, and consisted of Primer Set 1 (for gender determination and repeat sizing) and Primer Set 2 (for screening of expanded alleles). Each assay was evaluated for accuracy, precision, correlation with previous results, and workflow. **Results:** Both methods performed equally well in accuracy (+/1 repeat) compared to NIST standards and Coriell samples. Precision studies showed similar results for both systems (+/-0.04 to 0.12 repeats SD from mean). Both methods correlated with expected results for the selected Coriell samples from a previous consensus study, and showed 100% concordance with the genotype of previously analyzed patient samples. The Asuragen method was able to detect full mutation mosaicism down to 5% and premutation mosaicism to 1%. The Abbott Primer Set 2 was able to detect both full mutation and premutation mosaicism down to 25%. Neither method reveals methylation status. The most significant difference between the two methods is the workflow. The Asuragen method includes PCR followed by a single injection and analysis with capillary electrophoresis. The Abbott method includes two PCR reactions. The PCR product from Primer Set 2 (screening) can be analyzed directly by capillary electrophoresis, while the PCR product for Primer Set 1 (sizing) is first checked by agarose gel electrophoresis, enabling visualization of full mutation products as smears, followed by post-PCR clean-up and two injections on capillary electrophoresis (one for short and one for long fragments). **Conclusions:** The two PCR-based methods that we evaluated for the determination of *FMR1* mutation status are concordant with expected results in their final diagnoses and significantly differ only in their workflow.

G21. Clinical and Molecular Characterization of a Korean Patient with 9q22.3 Microdeletion Detected by Array Comparative Genomic Hybridization

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Introduction: Chromosomal disorders are often suspected in patients who display developmental delay, mental retardation, or dysmorphic features. However, the presence of submicroscopic genomic deletions or duplications cannot be detected by conventional chromosomal analysis. Array comparative genomic hybridization (CGH) is a newly developed molecular cytogenetic technique that enables the identification of copy number alterations in the human genome. Here we report a 9p22.3 microdeletion by array CGH in a child with dysmorphic features. The 9q22.3 microdeletion is recently proposed syndrome characterized with overgrowth, psychomotor delay, and distinct facial features including trigonocephaly, epicanthic folds, small mouth with thin upper lip, low set ears with ear lobule uplift. **Methods:** G-banded chromosome analysis was performed in the patient. Array CGH using human 135K whole-genome arrays (Roche NimbleGen, Germany) was also performed, and the results were analyzed using Genoglyphix software (Signature genomics, USA) and the online genome databases. The clinical and molecular findings were considered the implications through review of literatures published about 9q22.3 microdeletion. **Results:** Chromosome study showed that the patient had 46,XX,t(9;10)(q22.3;q11.2). And array CGH revealed a 4.7 Mb interstitial deletion involving 9q22.31-q22.33 region. She had similar findings with 9q22.3 microdeletion including overgrowth, facial dysmorphism, and hypotonia. A female infant was delivered at 36 weeks of gestational age by vaginal delivery. At birth, she was large for the gestational age and showed macrocephaly with a birth weight of 2920g (>95%), length 51cm (>95%), and head circumference 36cm (>95%). Dysmorphic features included frontal bossing, small mouth with thin upper lip, low set ears with ear lobule uplift and short neck. Brain MRI and cardiac echocardiography showed mild ventriculomegaly and patent ductus arteriosus. The neonatal period was complicated by respiratory difficulties due to respiratory distress syndrome, feeding difficulties and hypotonia. Besides the deleted portion in the patient contains PTCH1 gene responsible for nevoid basal cell carcinoma syndrome (NBCCS, Gorlin syndrome, OMIM #109400) and thus the patient should be observed carefully. **Conclusions:** In

the present study, we identified a 9q22.3 microdeletion in a child with overgrowth and dysmorphic features. To the best of our knowledge, this is the first case of 9p22.3 microdeletion in Korea. The result from our study demonstrates that array CGH is a useful method for detection of subtle genomic imbalance and prediction of clinical outcome. Further analyses such as FISH are needed to validate this study and a long-term follow-up of the patient would contribute to extend our understanding about 9q22.3 microdeletion.

G22. Clinical Application of Warfarin Pharmacogenetics (PGx): the Experience of the Philadelphia Veterans Affairs Medical Center (PVAMC)

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Introduction: Warfarin is a major cause of adverse drug reactions and morbidity. Single nucleotide polymorphisms (SNPs) in the *CYP2C9* and *VKORC1* genes affect warfarin metabolism and anti-coagulation response, and have been associated with dosing requirements and outcomes of warfarin therapy. Clinical use of warfarin PGx correlated with decreased hospitalization rates in a recent prospective study. While ongoing large-scale randomized studies may ultimately determine the cost-effectiveness of routine warfarin PGx, several clinical groups are using PGx to help guide therapy or explain atypical therapeutic responses. We report the experience of the PVAMC, where warfarin PGx has been available for clinical use since June, 2008. **Methods:** PGx was requested in 49 patients with difficulties in establishing a stable warfarin dose. Patients were followed by PharmD providers in the Anticoagulation Clinic and included 48 males, 26 Caucasian, and 23 (47%) African-American patients. DNA was extracted from EDTA-anticoagulated blood using EasyMag (Biomerieux) and analyzed by microarray using the *CYP2C9-VKORC1* assay and the Autogenomics Infiniti analyzer. Clinically relevant SNPs measured include *CYP2C9 *2-6, *11*, and *VKORC1* 3673. Patients had 4-51 visits for potential dose adjustments. The last prescribed warfarin dose was compared to the PGx-predicted dose according to the *http://www.warfarindosing.org* algorithm. An interpretative note including PGx-guided dose recommendations was created by a pathologist on the patient's electronic medical record. Linear regression and analyses of variance with Holm-Sidak correction were used for statistical testing. **Results:** *CYP2C9* variants were identified in 15 (31%) patients: homozygous *2 (1), and heterozygous *2 (4), *3 (5), *5 (1), *6 (2), and *11 (2). *VKORC1* genotype distribution at the 3673 site was as follows: A/A (4), G/A (16), and G/G (29). African-Americans comprised 72% of the patients with the G/G genotype, while Caucasians accounted for 88% of G/A and 75% of A/A. There was a good correlation between PGx-predicted dose and last prescribed dose (R2=0.60). Patients with 3673 G/G, a variant associated with warfarin resistance, and wild-type *CYP2C9*, had higher final dose requirements ($p<0.001$), larger increases from initial dose ($p=0.005$), and wider ranges of dose adjustments ($p=0.029$), as compared to other genotypes. **Conclusions:** These data suggest that PGx-guided warfarin dosing recommendations may be useful even in a well-managed anticoagulation clinic environment with close patient follow-up, particularly when a large proportion of African-American patients results in high prevalence of warfarin resistant genotypes. Effective communication between the laboratory and the anticoagulation providers is essential for proper interpretation of warfarin PGx results.

G23. High Prevalence of Simultaneous Factor V Leiden, Prothrombin G20210A and *MTHFR* C677T Mutations in Lebanese Patients Referred for Thrombophilia Workup

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Introduction: Molecular diagnostics has markedly improved the diagnosis and work-up of different clinical conditions including hypercoagulable state or thrombophilia where different genes are involved. In this report, which is the largest report in the medical literature and the first in Lebanon, we describe the prevalence of simultaneous mutations in the three major thrombophilia genes, Factor V, Factor II, and Methylenetetrahydrofolate reductase. **Methods:** Study Samples and DNA Extraction: we analyzed 2248 cases referred for thrombophilia workup at the American University of Beirut Medical Center. The patients, based on the specialty of the referring physicians, were mainly referred for work-up of recurrent abortions, deep vein thrombosis (DVT), stroke, pulmonary embolism (PE) and catheter thrombosis between January 2003 and February 2011. Their DNA was originally extracted using the PEL-FREEZ extraction kit (PEL-FREEZ, DYNAL, USA) and stored at -80°C for later use. PCR and Reverse Hybridization: To test for the various genotypic profiles of the Factor V, Prothrombin, and Methylenetetrahydrofolate genes, the FV-*PTH-MTHFR* StripAssay (ViennaLab, Austria) was used and the manufacturer's protocol was followed as recommended. This assay screens for the G1691A, G20210A, and C677T mutations of the Factor V, Prothrombin, and *MTHFR* genes, respectively, whereby *in vitro*, the different gene sequences are simultaneously amplified and biotin-labeled in a single amplification reaction. **Results:** Out of the 2248 referred patients, 25 (1.11%) had Factor V, Factor II, and *MTHFR* gene mutations simultaneously. As deduced from the referral diagnosis stated by the physician, 13 patients out of the 25 had DVT, 5 had recurrent abortions, 4 had stroke, 2 patients experienced catheter thrombosis, and 1 patient had both DVT and PE. **Conclusions:** With this presumably high prevalence rate of triple mutations, the Lebanese community again shows a more peculiar and specific finding for this special population and may label the Lebanese people as "more prone" for thrombotic disorders. We highly advocate for clinical studies to be performed at a larger scale in Lebanon (as well as other countries) for better correlation with the genetic findings based on this and other national and international studies.

G24. Combined Sanger- and Pyrosequencing in the Molecular Evaluation of Dysfibrinogenemia

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Introduction: Fibrinogen, a plasma glycoprotein synthesized by the liver, is an essential component of the blood clot. The mature protein is composed of three subunits, encoded by one of three homologous genes on Chromosome 4q: fibrinogen alpha (*FGA*), fibrinogen beta (*FGB*) and fibrinogen gamma (*FGG*). More than 40 mutations in *FGA*, *FGB* and *FGG* have been reported in autosomal dominant dysfibrinogenemia, a disorder defined by normal levels of functionally abnormal plasma fibrinogen. Patients with dysfibrinogenemia may remain clinically asymptomatic or present with bleeding and/or thrombosis. Dysfibrinogenemia is screened in the laboratory through a combination of prolonged thrombin time and reptilase time and decreased fibrinogen activity/antigen ratio. However, these tests do not distinguish between acquired and inherited forms of fibrinogen dysfunction and neither predict the recurrence risk nor assist in the decision to institute therapy. We hypothesize that genetic analyses of fibrinogen genes can be useful in diagnostic confirmation and in predicting the clinical presentation of inherited dysfibrinogenemia. **Methods:** Seven subjects were consented on an IRB-approved protocol at Texas Children's Hospital based on either abnormal screening tests, or history of unexplained bleeding and/or thrombosis. Five members of a multigenerational family, including four with abnormal screening tests and one unaffected member, and two additional probands were recruited. Controls were unaffected family members or unrelated individuals without laboratory abnormalities. PCR and direct Sanger sequencing was performed in duplicate on genomic DNA extracted from peripheral blood using primers amplifying all 6 coding exons of *FGA*. Separate PCR and pyrosequencing primers, designed to amplify and confirm hot-spot mutations in exon 2 of *FGA* were evaluated using PyroMark PSQ-Gold SQA reagents on the PSQ-96MA pyrosequencer (Qiagen Inc., CA) **Results:** Mutations were identified in *FGA* in all six cases with either positive history or laboratory evidence of dysfibrinogenemia. Whereas the unaffected relative was wild type, all four affected members of the single family were heterozygous for the c.104G>A (R35H) mutation that affects thrombin-mediated fibrinopeptide A release. These patients may have an increased risk of spontaneous abortions and postpartum bleeding. The remaining two patients were heterozygous for known deleterious mutations c.103C>T (R35C) and c.112A>G (R38G). Each mutation was confirmed in a separate pyrosequencing assay. The molecular analyses showed perfect correlation with the laboratory abnormalities. **Conclusions:** Molecular analyses of suspected dysfibrinogenemic patients using a combination of traditional sequencing and pyrosequencing can be a useful adjunct tool in providing diagnostic confirmation, and general risk assessment for selected well-characterized mutations.

G26. Hereditary Amyloidosis: More than Just Transthyretin

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Introduction: Mutations in transthyretin (*TTR*) account for 95% to 98% of hereditary amyloidosis. Transthyretin synthesis occurs in the liver, and phenotypic manifestations are peripheral polyneuropathy and/or cardiomyopathy, with or without eye or brain involvement. Therapeutic management varies based on the origin of the amyloid protein, with liver transplantation implemented in individuals with *TTR* amyloidosis. However, at least 2% to 5% of hereditary amyloidosis is due to aberrantly folded protein products of other genes. Some of these other genes also have potential founder effects, genotype-phenotype correlations, and specific therapies. In order to capture the remaining genetic etiologies for hereditary amyloidosis, we developed gene sequencing assays to confirm suspected mutations in additional genes, some of which were detected by tissue laser-capture tandem mass spectrometry (LC MS/MS) in patients with this presumed clinical diagnosis. **Methods:** Sequencing assays using universal primer sequence tags were developed for whole blood specimens for the following genes: *ApoA*1, *ApoA*2, Fibrinogen Alpha (*FGA*), GelsolinA (*GsnA*), and Lysozyme (*Lys*). To design the primers, each gene was analyzed in Alamut® v1.5, and the coding

sequence was obtained from the UCSC genome browser website. Potential SNPs were obtained using the Oligo Typer Analyzer program (Mayo Clinic, Rochester). Each primer was analyzed for melting temperature, potential hairpins, and other features. The assays were validated on 22 samples as follows: *ApoA*1 (9); *FGA* (7); *GsnA* (5); *Lys* (1). Samples were obtained from our LC MS/MS lab (3), Mayo Clinic research laboratories (5), and outside research laboratories (14) for both intra- and interassay precision and accuracy. **Results:** We identified pathogenic changes in three cases of non-*TTR* familial amyloidosis detected by LC MS/MS. Mutations were confirmed in the 19 cases with known variants. The repeat rate due to signal intensity or base call quality issues was less than 5% for four of five genes. *ApoA*2 required a second set of primers that decreased the repeat rate to 11% for raw signal, and 3% for quality. **Conclusions:** In each case, the sequencing primers designed for each respective gene successfully identified known pathogenic mutations. Clinically, identification of the genotype for amyloidosis is important to properly direct the management of the patient and for genetic counseling for the family. These tests will provide valuable information for cases in which less common familial amyloidoses are identified, either clinically, or by advanced laboratory methods such as LC MS/MS.

G27. Effective Detection of Germline Copy Number Variations of Cancer Predisposition Genes Using a Whole Genome Array with Exon Coverage of Disease Genes

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Introduction: Constitutive (germline) genetic variations have been associated with increased risk of cancer with or without congenital defects. Some of the detected variations are copy number variations (CNVs). **Methods:** We have been using customized oligonucleotide-based arrays for the detection of CNVs associated with genetic diseases by chromosomal microarray analysis (CMA). Our clinical array allows the interrogation of not only the entire genome but also exon coverage of more than 1700 disease or candidate genes including over three dozen genes relevant to cancer family syndromes. **Results:** Out of >25,000 patients referred to for CMA testing, we identified 59 patients with germline copy number losses of cancer predisposition genes. The involvement of cancer risk genes are unexpected in most cases except for some cases referred for neurofibromatosis, WAGR, and retinoblastoma. A small deletion containing only part of a single gene were identified in six patients including three patients having a deletion within the *PMS2* gene of ~1 kb, ~4 kb, and ~10 kb in size, and the other three patients having a ~8 kb deletion of the exon 3 to 5 of *PTEN*, a ~12 kb deletion of the exon 3 of *SDHB*, and a ~41 kb deletion of the exon 41 to 58 of *NF1*, respectively. One patient has a ~ 4 kb deletion including the exon 5 to 10 of *MSH6* and the 3' untranslated region of an adjacent gene. The remaining patients have a deletion of >0.9 Mb in size containing multiple genes. The copy number losses in three cases are part of a complex rearrangement. Of the 20 unrelated patients with parental information, the deletions in ~85% (17/20) patients are de novo and the remaining ~15% (3/20) rearrangements are maternally inherited. These inherited rearrangements include the two small deletion involving *SDHB* or *MSH6* and a recurrent 0.9 Mb deletion involving *TMEM127* in 2q11.2 identified in two siblings. **Conclusions:** Our data demonstrated that array CGH using a high density whole genome array are effective in detection of large copy number changes spanning the entire cancer predisposition gene that may be undetectable by DNA sequencing. In addition, exon-by-exon coverage of disease genes allows small variations involving only part of a single gene to be detected. Because most of these CNVs are accidental findings in patients referred for developmental defects, cancer genetic counseling and parental studies are needed for cancer surveillance and recurrent risk estimation.

G28. A 106 Mutation Cystic Fibrosis Laboratory Developed Testing Using the Sequenom MassARRAY®: Performance in the First 18,000 Tests

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Introduction: In 2001, the ACOG and ACMG recommended population-based carrier screening for cystic fibrosis (CF) using a panel of 23 mutations. Since that time, some have argued that expanded panels with additional mutations would be useful in non-Northern European populations. We co-developed a CF screening test with Sequenom, Inc. that targets 106 mutations. Analytical verification of the assay has been presented (*JMD* 2010, 12:611-619). **Methods:** The Mass Array CF screening test was validated according to CLIA guidelines. Since validation, the Molecular Genetics Laboratory has tested 18,559 clinical samples. The workflow consists of 8 multiplex PCR reactions, removal of dNTPs and primers with AMPure resin (Beckman-Coulter), 8 multiplex single base extension (SBE) reactions, desalting with an ion exchange resin, spotting the extended SBE products on a MassARRAY® chip, and analysis by MALDI-TOF mass spectrometry. 48 samples and controls are run per batch in 384 well plates. All steps are automated, and the test requires 9 hours to complete. **Results:** Of the clinical samples tested, 586 were for a possible or known diagnosis of CF, pancreatitis, or male infertility; the remainder had indications for testing of routine carrier screening, a family history of CF, a partner that was a known CF carrier, or no indication given. Of the total number of samples tested, 821 (4.4%) had at least one mutation. Of the 124 mutations detected in the diagnostic cases, 114 (91.9%) were ACMG panel mutations, 78 of which were dF508. Of the 695 mutations identified in samples tested for carrier status, 627 (90.2%) were ACMG panel mutations (443 dF508) and 68 (9.8%) were non-panel. Two mutations were identified in one individual with a referral for carrier screening. The genotype for this individual was dF508/R117H-7T. Two mutations were detected in 3 individuals with no indications for testing provided. In two cases, the 2nd mutations were known to be associated with mild disease, 3849+10kbC>T and D1152H. One individual was homozygous for 3849+10kbC>T. We have detected 46 different mutations, including 21 of the 23 mutation ACMG panel (711+1G>T and R334W have not yet been detected). We have identified 2 mutations that are not on any other currently available panel. **Conclusions:** Of all detected mutations, nearly10%, were non-ACMG panel mutations, indicating that a significant fraction of the population can benefit from screening with an expanded panel.

G29. Comparison of Extracted DNA vs. Non-Extracted Sample (Direct) for the Detection of Factor V Leiden, Factor LI, And *MTHFR* Single Nucleotide Polymorphisms from Whole Blood and Buccal Swabs

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Introduction: Molecular methods traditionally entail extraction of nucleic acids prior to performing amplification and detection steps. We have developed reagents that enable direct sample-to-answer detection of nucleic acids from various specimen types without performing any nucleic acid isolation or purification steps. We used reagents to detect factor V Leiden, factor II, and methylenetetrahydrofolate reductase (*MTHFR*) single nucleotide polymorphisms (SNPs) from whole blood and buccal swab samples, and compared methods using nucleic acid extraction to the direct detection methodology. **Methods:** Whole blood (previously tested by a reference method) and buccal swab samples were evaluated. Whole blood was collected using EDTA, sodium citrate, or sodium heparin as an anticoagulant. Real-time PCR assays were performed to compare extracted DNA vs. extraction-free (direct) amplification of these samples. The master mix utilized for direct testing was optimized to provide robust amplification directly from specimens, even in the presence of potential inhibitory substances. **Results:** Genotypes obtained for both alleles were in 100% agreement with those obtained by the reference method. In addition, complete concordance was obtained between extracted samples and those that were amplified directly without extraction. Direct detection was achieved with both whole blood (in all anti-coagulants; EDTA, sodium citrate, and sodium heparin) and buccal swab samples, and none of the samples showed inhibition. Testing time (from sample to result) was less than 40 minutes for both sample types. **Conclusions:** The ability to directly amplify and detect nucleic acids without previous extraction and purification processes demonstrates the robust nature of the amplification chemistry. In addition, accurate direct detection and discrimination of various SNPs was achieved from both sample types.

G30. CNV and Incomplete Linkage Disequilibrium Interference with the HCP5 Genotyping Assay for Abacavir Hypersensitivity

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Introduction: Abacavir sulfate (Ziagen®) is an effective antiretroviral drug used to manage HIV infection, but 5% to 8% of patients develop abacavir hypersensitivity reaction (ABC-HSR). ABC-HSR is a life threatening condition that is ethnic-dependent and associated with the human leukocyte antigen *HLA-B*5701* allele. Current guidelines for antiretroviral treatment recommend screening for *HLA-B*5701* prior to initiating abacavir therapy. However, HLA typing or sequencing remains prohibitively expensive for routine screening. In Caucasians a SNP (rs2395029) in the major Histocompatibility Complex P5 (*HCP5*) gene, is reported to be in linkage disequilibrium (LD) (r2=1) with the *HLA-B*5701* allele. Genotyping for *HCP5* has been increasingly adopted as a simple, inexpensive method to screen for ABC-HSR. In this study we evaluated the genotype concordance between the *HCP5* SNP and *HLA-B*5701* allele in a large sample set. **Methods:** 1,889 DNA samples were genotyped for the *HCP5* polymorphisms by real-time hybridization probe assay and for the *HLA-B*5701* allele by PCR with sequence specific primers (PCR SSP). **Results:** Overall, a good correlation between the two genotyping methods was found with analytical sensitivity (0.99%) and specificity (0.99%). Interestingly, the *HCP5* SNP could not be amplified in two samples, both negative for *HLA-B*5701*. Further investigations with a custom comparative genomic hybridization (CGH) array demonstrated that both samples were homozygous for large deletions including the *HCP5* gene. The Database of Genomic Variants shows

that this region encompassing the *HCP5* gene is the site of a recurrent copy number variation (CNV), and that does not extend to the HLA-B locus. In addition, nine samples with discordant genotyping results between the two methodologies were identified. These included: eight samples *HLA-B*5701* negative/*HCP5* positive, and one sample *HLA-B*5701* positive/*HCP5* negative. **Conclusions:** *HCP5* genotyping results showed a good correlation with *HLA-B*5701* typing by PCR SSP. However we have detected two samples with homozygous deletions of the *HCP5* gene and found the locus is the site of a CNV that does not include the HLA-B locus. The fact *HCP5* occurs within a region of CNV and the possibility that linkage disequilibrium between the *HLA-B*5701* and *HCP5* SNP may vary between ethnicities is a concern. The possibility of incomplete disequilibrium and CNV should considered, particularly when *HCP5* genotyping is performed in patients who are not of European ancestry.

G31. Frequency of 3' Deletions in *PMS2*

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Introduction: Lynch syndrome is characterized by mutations in one of four mismatch repair genes, *MLH1*, *MSH2*, *MSH6*, or *PMS2*. While the detection of mutations in *PMS2* is greatly complicated by numerous pseudogenes, long-range PCR can be employed to specifically amplify the gene and detect sequence changes. For detection of large deletions, multiplex ligation-dependent probe amplification (MLPA) has been employed for exons 1 – 11. We have recently described an MLPA-based method that avoids *PMS2CL*, a pseudogene with extensive homology to the 3' end of *PMS2*, and now permits detection of deletions for this region of the gene (exons 12 – 15) as well. However, the frequency of 3' deletions of *PMS2* has not yet been determined. **Methods:** Sixty-three samples for which immunohistochemical staining suggested a *PMS2* mutation, but for which no mutation was identified using previously available methods, were evaluated for 3' deletions in *PMS2* using our new method. This method utilizes MLPA probes for *PMS2* and *PMS2CL* with the selection of appropriate reference samples and sequencing the gene and pseudogene in this region. **Results:** Evaluation of this cohort of samples identified six samples with deletions in the 3' region of the gene, including three previously reported samples with deletions in intron 12 – exon 15, exons 13 – 15, and exons 14 – 15. Of the additional three samples with deletions, one sample harbored an exon 13 deletion and two samples harbored exon 14 deletions. Overall, 6/63 (9.5%) samples in which *PMS2* mutations had not been identified by existing methodologies harbored deletions in the 3' region of *PMS2*. **Conclusions:** These results indicate that ~10% of samples suspected of harboring a *PMS2* mutation based on immunohistochemical staining, for which mutations have not yet been identified, may benefit from testing using the new methodology.

G32. Rapid Single-Tube Genotyping of 23 *CFTR* Mutations Using High Resolution Melt Analysis (HRMA)

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Introduction: Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene are responsible for cystic fibrosis and *CFTR*-related disorders. Both can affect multiple systems/organs in the body, including the lungs, pancreas, intestines, and liver. In the non-Hispanic white population CF disease incidence is estimated at 1 in 2500. Though the incidence is less in other ethnic populations, ACOG has recommended carrier and prenatal screening as part of routine practice because it has become increasingly difficult to assign a single ethnicity to affected individuals. Molecular diagnostics are routinely used for carrier screening and for diagnosis confirmation of other positive/borderline CF newborn screening tests. Commercially available tests can take up to 8 hours to perform and require several tube transfers. We have developed a rapid single-step detection method to screen for the 23 *CFTR* mutations recommended by ACOG using HRMA. Small amplicon assays were designed to genotype 18 of the 23 loci of interest. The remaining 5 mutations (A455E, ΔI507/ ΔF508, G551D/R553X) employ unlabeled probes following amplification for genotyping. In addition to genotyping disease-associated mutations this method can identify benign variants known to interfere with the identification of ΔF508 or ΔF507 mutations. **Methods:** PCR reactions were amplified on the Roche LightCycler®480 system in the presence of LCGreen®Plus dye. Eighteen targets used routine amplification conditions and 5 targets were amplified using asymmetric PCR and had an additional probe that was blocked from participating in amplification. HRMA was performed at a rate of 0.6°/sec on all amplicons or amplicon/probe mixes immediately following PCR in the same tube in the same instrument. HRMA data was downloaded to in-house developed software or MeltWizard (courtesy of Carl Wittwer) for pattern analysis. Thirty-two genomic DNAs acquired from the Coriell Institute and one from patient DNA were tested for each target. Thirteen synthetic plasmid DNA constructs specifically designed for the targeted regions were also tested. Expected amplicon size was confirmed using the Agilent Bioanalyzer instrument. Genotypes were called based on pattern analysis. **Results:** All 33 genomic DNAs and 13 synthetic DNAs showed the expected genotype with HRMA. Challenge with synthetic DNAs that represent benign mutants demonstrated patterns that could be distinguished from the clinically relevant ΔF508 or ΔI507 mutations. **Conclusions:** We have demonstrated a rapid sensitive method for detection of CF mutations. In addition, this method can easily distinguish clinically relevant ΔF508 or ΔI507 variants from benign variants F508C, I506V, and I507V.

G33. Germline Mutations in *MLH1* and *MSH2* in Korean Hereditary Non-Polyposis Colorectal Cancer (HNPCC) Patients

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Introduction: Hereditary non-polyposis colorectal cancer (HNPCC), the most common hereditary colon cancer syndrome, is an autosomal dominant disorder with defects in mismatch repair (MMR) system. Identification of MMR gene mutations can have direct clinical implications in management and counseling of HNPCC families. We tested 27 suspected HNPCC Korean patients for germline mutations in two MMR genes, *MLH1* and *MSH2*. **Methods:** Suspected HNPCC patients were enrolled according to the clinical manifestation and Amsterdam criteria II in Asan medical center. Tumors were tested for *MLH1* and *MSH2* immunohistochemical staining (IHC) and microsatellite instability (MSI), a hallmark of HNPCC. Germline MMR gene mutations (*MLH1* and *MSH2*) were tested with PCR direct sequencing method covering all the coding regions. The remaining mutation-negative cases were analyzed using multiple ligation dependent probe amplification (MLPA). Clinical data were obtained by chart audit. **Results:** Among 27 suspected HNPCC patients, four patients have *MLH1* gene mutations: c.1758dupC, c.1721T>C, c.666T>G and c.2101C>A. Eight patients have *MSH2* mutations: c.2361dupT, c.1024delinsAA, c.1147C>T, c.2068C>T, c.2633_2634delAG, c.-225G>C, c.2006G>A and c.1168C>T. Three mutations (c.2361dupT, c.1024delinsAA, c.2068C>T) in the *MSH2* gene have not been reported elsewhere. There was no patient whose MLPA results were positive for *MLH1* or *MSH2*. **Conclusions:** In this study, 4 patients (14.8%) had *MLH1* gene mutations and 8 patients (29.6%) had *MSH2* mutations. We identified three novel mutations in *MSH2* gene. Identification of *MLH1* and *MSH2* mutations is important for predictive diagnosis and clinical management of HNPCC patients. We described the incidence and distribution of MMR gene in Korean HNPCC patients. Also, our results could broaden the spectrum of *MLH1* and *MSH2* mutations by identifying three novel mutations. Further investigation including systematic familial studies with larger cohorts of patients should be conducted.

G34. Investigation of Exon-Focused Array CGH as a Diagnostic Tool for Copy Number Mutations in Mendelian Disorder

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Introduction: Copy number changes including deletion and duplication mutations in human genome have been known as important cause of Mendelian disorders. Recently major advancement in detection of copy number change has been achieved by development of microarray technique. The aim of this study is to explore the possibility of exon-focused microarray CGH as diagnostic tools of Mendelian disorders by gene copy number change. **Methods:** Diagnostic usefulness of exon-focused microarray CGH was investigated about 87 Mendelian disorders using the NimbleGen Human CGH 3*720K Whole-Genome Exon-Focused Array (Nimblegen, Roche, USA). Copy number change was measured about 7 healthy controls and 14 cases that showed copy number change by MLPA methods, and Probe signal stability was measured by coefficient variance (CV)% of probe signal ratio about each genes. In addition, we compare the probe composition of MLPA and exon-focused array CGH about 4 disease categories that include Charcot Marie-Tooth/Hereditary Neuropathy with liability to Pressure Palsies Disease, Von Hippel Lindau Syndrome, Spinal Muscular Atropy and Duchene Muscular Dystrophy. **Results:** Among 87 genes, 75 showed CV less than 10% and 49 genes had 1 more probes in all exon. Genes with pseudogenes including *SMN*, *SMN2*, *CYP21A2*, *PMS2*, and *PRSS1* showed high CV% and also gene dosage showed difference with expected results. In case of genetic disease that showed mutation pattern with whole gene deletion or duplications, exon focused array CGH detected the accurate gene dosage. In case of copy number mutation with exonic level, copy number changes of two more exons were detected by exon focused array CGH. However, copy number change in single exon was detected by modification of analysis program algorism. **Conclusions:** Exon-focused array CGH method was considered as useful diagnostic tools for the detection of Mendelian disorder by gene dosage mutation because in had much more probe numbers than MLPA and stable fluorescence signal

ratio about each probes. But it could not measure the accurate gene dosage in the presence of pseudogenes.

G35. Fragile X Carrier Screening and Allele Distribution of the *FMR1* Gene in Korean Women

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Introduction: Fragile X syndrome (FXS), the most common form of familial mental retardation, is caused by the expansion of CGG sequences in the 5' untranslated region of the *FMR1* gene on the X chromosome. Previous studies have suggested that prevalence of FXS in Korean population was lower than that observed in Caucasians. In addition, no premutation alleles were found in a previous screening among the general Korean women. The aim of this study was to investigate the prevalence of carriers of premutation-size alleles and the distribution of the *FMR1* alleles in Korean women. **Methods:** The study subjects were Korean women without mental retardation from January 2007 to April 2011. We analyzed a total of 20,023 normal female subjects and attempted to estimate the frequency of the *FMR1* allele. Their CGG repeats were determined by using PCR and fragment analysis. **Results:** Among a total of 40,046 alleles, 39,952 alleles (99.77%) were within the normal range. The number of normal CGG repeats ranged from 8 to 44, with a modal number of 28 (39.06%), a second peak at 29 (25.69%). 11 subjects were shown to have a premutation allele (55-73 repeats). Intermediate allele was defined as 45–54 repeats, and a total of 83 alleles were found within this range (0.21%). **Conclusions:** This is the first large study focusing on the prevalence of FXS and the *FMR1* allele distribution by analyzing the normal, intermediate and premutation alleles in the Korean female population. In this study, the prevalence of carriers of premutation-size alleles in Korean women can be estimated to be 1 in about 2,000.

G36. *FLCN* Mutations in Korean Patients with Primary Spontaneous Pneumothorax

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Introduction: *FLCN* gene is introduced to a causative gene of primary spontaneous pneumothorax (PSP). We investigated *FLCN* mutations in Korean patients with PSP. **Methods:** The 48 patients with PSP were enrolled in this study. DNA was extracted from formalin-fixed paraffin embedding lung tissue. We amplified and sequenced each exon and the adjacent intron from exon 4 to exon 14 using previously reported primers. **Results:** Four mutations that were c.712G>A in exon 7, c.880G>A in exon 9, c.1223A>G, and c.468_470delTTC in exon 11 were revealed. The 23 genetic variations with one nucleotide change were identified in exons and introns. **Conclusions:** Unlike previous Caucasian studies, nonsense mutation and frameshift mutation in genomic DNA level is relatively low incidence in Korean patients with PSP.

G37. Novel Alternative Splicing of *FLCN* Gene

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Introduction: *FLCN* gene is related with primary spontaneous pneumothorax (PSP) and several tumors. We identified a novel alternative splicing of *FLCN* gene and investigated its clinical significances. **Methods:** The PSP patients (n=10) and normal persons (n=20) were enrolled in the study. Genomic DNA and total RNA were extracted from venous blood sample, and cDNA was synthesized. We amplified and sequenced each exon and the adjacent intron in genomic DNA level. The entire coding region in mRNA was amplified, sequenced and was cloned. We sequenced the entire cloning cDNA region from 20 colonies per each sample. Long PCR and direct sequencing from exon 2 to exon 5 were performed in genomic DNA. For identifying proportion of mRNA with alternative splicing, we performed a colony PCR from 96 colonies per each sample using primers for amplification of deletional region, and we also performed a real-time ddCt analysis using alternative splicing-specific primers and alternative splicing-nonspecific primers. **Results:** In mRNA level, *FLCN* c.-113_-25del89 in exon 3 was identified. However, this variation and any nucleotide changes in junction region of exon 2 to exon 4 according to the reference sequence (NM_144606) were not observed in genomic DNA level. In colony cDNA sequencing, mRNA with exon 3 deletion had the heterzygotic evidences identified in exon region of genomic DNA level. In colony PCR and real-time ddCt analysis, proportion of mRNA with total deletion of exon 3 induced by alternative splicing was about 30%. Above findings were similar between the PSP patients and normal persons. **Conclusions:** Novel alternative splicing of *FLCN* gene may be a normal variant in blood cells.

G38. Identification of a Small 15q13.3 Deletion in Several Patients: Further Evidence Implicating *CHRNA7* in the 15q13.3 Deletion Phenotype

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Introduction: The 15q13.3 microdeletion syndrome is a recently described disorder characterized by highly variable presentations, which may include intellectual disability, cardiac malformations, seizures, autism, and psychiatric conditions such as schizophrenia or bipolar disorder. This deletion is inherited in approximately 75% of cases and has been found in mildly affected and normal parents, suggesting incomplete penetrance. The common deletion size is approximately 2 Mb and contains several genes; however the gene(s) responsible for the resulting clinical features have not been clearly defined. Recently, a few patients were described with a smaller, approximately 680 kb deletion including only the *CHRNA7* gene and, in some cases, a portion of the OTUD7A gene. These patients demonstrated a wide range of neurodevelopmental symptoms similar to those observed with the larger 15q13.3 microdeletion including mental retardation, developmental delay, and seizures. **Methods:** Array comparative genomic hybridization (array CGH) testing, using either an Agilent 44k or 180k oligonucleotide array, was used to identify clinically relevant gains or losses. Data was compiled from a database of patient samples tested in the Mayo Clinic Cytogenetics Laboratory from 2008-2011. Of these cases where a deletion of *CHRNA7* was detected, results were confirmed by fluorescent in situ hybridization (FISH) using the BAC probe RP11-106H13. **Results:** We identified an approximately 600 kb deletion including only the *CHRNA7* gene in 5 affected individuals. One of these patients was referred for a pervasive developmental disorder, while another (at age 5 weeks) had failure to thrive. The remaining three patients are family members (a brother and sister and their paternal first cousin) who are described as having cognitive deficits and/or developmental delays, and one child also has behavioral issues. The father of the siblings and the mother of their first cousin are siblings themselves and therefore are likely carriers; both have psychiatric problems. Confirmation testing for *CHRNA7* deletion in these individuals is pending. **Conclusions:** The 5 patients in our study, like those described previously in the literature, have a significant amount of phenotypic overlap when compared to patients with the larger 15q13.3 microdeletion. Therefore, these results provide further evidence implicating *CHRNA7* as the gene responsible for the neurodevelopmental phenotype spectrum resulting from 15q13.3 microdeletion.

G39. Comparative Study of xTag 60 Cystic Fibrosis Test v2 with CF InPlex® Panel Utilizing Archived and Freshly Extracted DNA

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Introduction: The xTAG Cystic Fibrosis 60 kit v2 (Luminex, Austin, TX) simultaneously detects and identifies a panel of 60 mutations and variants in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene in human blood specimens. This panel includes the mutations and variants currently recommended by the American College of Medical Genetics and American College of Obstetricians and Gynecologists (ACMG/ACOG.) The xTAG Cystic Fibrosis 60 kit v2 is a qualitative genotyping test that provides information intended to be used for carrier testing in adults of reproductive age, as an aid in newborn screening, and in confirmatory diagnostic testing in newborns and children. **Methods:** Genomic DNA was extracted from 79 whole blood samples using the Qiagen EZ1 XL or manual DNA extraction. Recently extracted DNA samples, archived DNA stored at -80°C, and rehydrated DNA samples stored in GenTegra DNA tubes (IntegrenX, Pleasanton, CA) were used in conjunction with the xTAG Cystic Fibrosis 60 kit v2 on the Luminex 200 system. All DNA samples were diluted to a concentration of 20ng/μl with nuclease free water. The xTAG CF60 kit v2 protocol was followed using Cycling Condition 1 for PCR and Allele Specific Primer Extension (ASPE) steps. Coriell cell lines were used as positive controls. All samples had been previously tested with the Hologic CF InPlex Invader assay (Hologic, Bedford, MA). **Results:** Of the 79 samples, 29 were normal, 48 were heterozygous, and 2 were homozygous mutant. Results showed that the xTAG CF 60 kit v2 produced accurate, reproducible results from -80°C archived DNA, rehydrated DNA and recently extracted DNA with no significant decrease in signal detection. There was also no overall increase in background signal. All controls gave the expected results and all results were concordant with those obtained from previous testing using the InPlex® CF Molecular Test. **Conclusions:** The xTAG Cystic Fibrosis 60 kit v2 with Cycling Condition 1 was able to detect up to 60 mutations with accuracy and precision from frozen archived, rehydrated archived, and recently extracted genomic DNA. Due to the expanded detection capability of the xTAG Cystic Fibrosis 60 kit v2, we were also able to detect mutations previously unresolved with the InPlex® CF Molecular Test.

G40. A 106 Mutation Cystic Fibrosis Laboratory Developed Test Using the Sequenom MassARRAY®: Analytical Validation

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Introduction: In 2001, the ACOG and ACMG recommended population based carrier screening for cystic fibrosis (CF) using a panel of 23 mutations. Since that time, some have argued that expanded panels with additional mutations would be useful in non-Northern European populations. We co-developed a CF screening test with Sequenom, Inc. that targets 106 mutations. Analytical verification of the assay has been presented (JMD 2010;12:611-9). **Methods:** The version of the test that was validated at Mayo Clinic differed from that described in 2 of the 106 mutations, the PCR clean-up, and the QC/resulting software. The test was validated according to CLIA guidelines. The accuracy was determined by running 664 samples (4% carriers) in parallel with the 70+6 Tag-It analyte-specific reagent set from Luminex, Inc.; a 43 sample, 27 mutation "challenge panel" that contained 7 potentially interfering variants; and a set of synthetic controls that contain 104 of the 106 mutations. Precision was assessed with 96 samples, 8 examples each of 11 mutations and wild type (WT), run daily for 3 days. Analytical sensitivity was assessed both by dilution a dF508 sample into WT and by testing the assay response with decreasing amounts of input DNA. Analytical specificity was determined by electrophoresis of all PCR products and by examination of the large number of WT samples run in the Accuracy study. **Results:** There were no discordances with the challenge panel or synthetic control panel. In the parallel analysis study, one sample had 3 mutations with a false positive Exon2,3del. In the 12x8x3 precision study, there were 3 discordant samples: 2 were due to transcription errors in the original result and one was due to a rare polymorphism at the SBE primer site. We were able to detect 10% dF508 alleles in a WT background. One mutation assay, 712-1G>T, required 10 ng of input DNA. All the other assays were robust to 2 ng of input DNA. All the PCR products gave single bands on PAGE, and no WT samples from the Luminex assay gave positive results. **Conclusions:** Accuracy, precision and analytical sensitivity and specificity were excellent. One false positive was noted, and current practice is to verify any Exon2,3del positives with MLPA. Of the 106 mutations targeted by the test, not all were included in the validation due to the rarity of the samples. Thus, our practice is to sequence verify the first two examples of any mutation not tested during validation.

G41. Effectiveness of an Expanded Mutation Panel Versus the ACMG Recommended Panel in Detecting *CFTR* Mutations in Pancreatitis Patients

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Introduction: As more non-geneticists are ordering genetic testing, the molecular diagnostic laboratory often plays a critical role in guiding the clinician to the most informative and cost-effective testing for their patients. Without this guidance, the clinician may order the largest mutation panel available just to cast a wide net ("more must be better"), without understanding the actual benefit (or lack thereof) of such testing. As an example, samples from the pancreatitis clinic at our institution have been sent out for *CFTR* mutation testing using a 97 mutation panel available at a commercial laboratory. We offer an FDA-cleared 32 mutation panel in-house. Both panels include the 23 most common mutations recommended by ACMG for cystic fibrosis mutation screening. **Methods:** To determine the efficacy of ordering the larger mutation panel for pancreatitis patients, we first performed a literature review to determine which mutations are typically seen in the *CFTR* gene in patients with idiopathic chronic pancreatitis. We then compiled the results from testing performed on patients between January 2009 and April 2011 to determine what mutations were identified by the send-out testing and whether those mutations would have been identified by our in-house 32 mutation panel. **Results:** The literature review revealed that most mutations that are identified in patients with pancreatitis are either present in the 23 mutation ACMG panel or in neither the 32 mutation panel nor the 97 mutation expanded panel. The analysis of our send-out testing revealed that between January 2009 and April 2011, a total of 81 samples were sent for *CFTR* mutation testing, of which, 71 samples (88%) were negative for the 97 mutations tested. Of the 10 positive samples (12%), 8 were heterozygous for a common mutation (4 samples with delta F508 and 1 sample each for G542X, N1303K, 3120+1G>A, R553X) and 2 samples were compound heterozygous (R117H/R560T, deltaF508/L206W). Twelve total mutations were detected in the samples that were sent out for testing, including 5 delta F508 mutations in both heterozygous and compound heterozygous samples. Of the 8 different mutations detected, only the L206W mutation is not included in our in-house 32 mutation panel. All of the other mutations detected are included in our panel. **Conclusions:** The analysis showed that there is little additional benefit from ordering an expanded *CFTR* mutation panel for pancreatitis patients. As a result, we are able to recommend initial testing with the 32 mutation panel, followed by sequencing if indicated.

G42. A Multi-Gene Assay Coupled with Bioinformatics: An Interdisciplinary Approach to Pharmacogenetics Testing

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Introduction: The rationale for the "Pharmacogenomic Resources for Enhanced Decisions in Care and Treatment" (PREDICT) initiative at our institution is to provide real-time genotype analysis and decision support to facilitate individualized drug therapy. Implementation of this initiative involved adoption of a mid-throughput multi-gene assay in the clinical laboratory. An interdisciplinary team approach was required and a bioinformatics infrastructure was essential for quality control (QC) reports and delivering patient results to healthcare providers in a timely, comprehensive, and secure manner. **Methods:** 2000 DNA samples primarily from patients in the catheterization lab were referred for testing using the VeraCode ADME (Absorption, Distribution, Metabolism, Excretion) Core Panel Kit (Illumina) and the BeadXpress plate reader (Illumina). The kit interrogates 185 loci across 34 genes. Cell line control DNA with known genotypes was used as positive controls on each 32 specimen plate. **Results:** Genotype results on 34 genes were generated, reviewed and securely stored. Currently, only *CYP2C19* variants are reported with associated decision support for Clopidogrel dosage. Sophisticated bioinformatics generate comprehensive QC reports to monitor for sample reproducibility, variant allele frequencies and locus performance. Reproducibility for Paragon and Coriell control DNA cell lines at 185 loci was 99.58% and 98.34%, respectively. A monthly QC plate was initiated to monitor the reproducibility of patient results by performing random repeat testing of patient specimens from the preceding month and checking for concordant results at all 185 loci. Thus far, 64 samples have been retested, with 98.67% concordance across all loci and 100% concordance for *CYP2C19* variants. Observed *CYP2C19* allele frequencies are similar to that previously reported from the Database of Single Nucleotide Polymorphisms (dbSNP). 15/185 loci are consistently "poor performers" failing to provide results in >95% of patient samples analyzed on each plate. **Conclusions:** Implementation of clinical testing for pharmacogenetic variants utilizing this assay requires stringent review of the results. The establishment and monitoring of multiple QC indicators is essential and helps to ensure the accuracy of the results reported by this test. The established bioinformatics infrastructure coupled with the genotype data generated from the ADME kit enables genetically informed medicine and allows expansion of the decision support process for drug dosing of other known allelic variants for genes included in this kit.

G43. Development of a Multi-Locus Coronary Heart Disease (CHD) Genotyping Assay and Assessment of Accuracy and Robustness

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Introduction: Current cardiovascular risk assessment algorithms fail to predict many cardiovascular events. The polygenic nature of cardiovascular disease and modest risk estimates of the SNPs associated with CHD make the case for aggregating genetic information into a risk score to provide a more compelling case for individualized patient management. We have developed a multiplex assay that examines 20 genetic polymorphisms that have been found to be associated with coronary heart disease in multiple well-powered studies. Many of the 20 SNPs are in genes that may be involved in the pathogenesis of CHD including cholesterol homeostatis, platelet function, thrombosis, cell cycle progression, cell migration, protein secretion, and inflammation. Several SNPs are in genes or chromosomal regions that have functions yet to be determined. Here we demonstrate the performance characteristics of this multiplex assay on remnant, de-identified clinical samples. **Methods:** DNA was extracted on an automated platform from 1 mL samples of de-identified blood from 500 individuals collected as part of routine clinical testing by Berkeley HeartLab (Alameda CA). After quantitation and normalization, the DNA samples were multiplex PCR amplified. An Oligonucleotide Ligation Assay incorporating custom sequence-tagged Luminex® beads and biotin/Streptavidin Phycoerythrin detection was used for the automated simultaneous determination of all 20 genotypes by generic research software that compares the ratio of fluorescent signals between the two alleles for each SNP. **Results:** The 500 DNA specimens were genotyped with the CHD multiplex assay at various DNA concentrations. Genotype call rates remained above 99% with as little as 0.3 ng input genomic DNA. Genotype call concordance remained at 99.99% over the range of 0.3 to 3.0 ng input genomic DNA. All 500 samples were also genotyped at 3.0 ng input DNA using another validated technology, allele-specific, real-time PCR. Call concordance was 99.93%, between the two technologies. **Conclusions:** We evaluated a multi-locus genetic assay that simultaneously detects 20 SNPs that have been found to be reproducibly associated with CHD. The study demonstrated a >99% call rate and genotype concordance when compared with allele-specific, real-time PCR genotyping. The assay requires as little as 0.3 ng DNA, an elapsed time of 7 hrs, hands on time of

85 min, and is readily amenable to high throughput automation. This CHD assay will facilitate the collection of allele frequency and genetic association data from additional studies of different risk and ethnic groups.

G44. A Fragile X Screening Program Utilizing the Amplidex *FMR1* and mPCR-CE Assays by Asuragen

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Introduction: Fragile X Syndrome is the most common form of inherited intellectual impairment and a known genetic cause of autism. Risk assessment and clinical diagnosis of the disease is defined by the number of CGG repeats and methylation status of the *FMR1* gene located at Xq27.3. Normal repeat sizes range from 15-44, grey zone from 45-55, premutation from 56-199, and full mutation greater than 200 CGG repeats. The risk of CGG repeat expansion occurs from the mother passing a premutation or full mutation gene to her offspring. Fragile X carrier screening has been increasing within the past few years. Here we discuss the importance of prenatal carrier screening. **Methods:** A total of 323 peripheral blood specimens submitted for prenatal screening were extracted utilizing the EZ1 Advanced XL (Qiagen, Valencia, CA) and tested utilizing the Amplidex *FMR1* assay (Asuragen, Austin, TX). Results were reported in CGG repeat size after capillary electrophoresis. Specimens with a pre- or full mutation were tested for methylation status utilizing the mPCR *FMR1* assay (Asuragen, TX). **Results:** At our institution, a total of 323 specimens for fragile X testing were submitted with an indication of prenatal carrier screening (with or without history of developmental delay) during a 9 month period. 319 were reported normal to grey zone range with no risk for expansion to full mutation in the fetus. Two specimens were reported in the premutation range with risk to full mutation. Both patients reported family history of learning disabilities. Methylation status revealed partial methylation of the *FMR1* gene in both cases. One specimen was reported as a full mutation with risk of carrying an affected fetus. Methylation status was analyzed as partially methylated in this sample. This patient reported no family history of developmental delay or learning disability. **Conclusions:** Although our small study revealed a risk detection rate of 1%, the detection of an undiagnosed full mutation carrier increased the awareness of the incidence of this disease in the general population. The utilization of the Amplidex *FMR1* PCR assay makes carrier screening easy and affordable to serve the prenatal population. Further analysis of pre and full mutations utilizing the methylation PCR assay delivers a full diagnosis to the clinician and patient, providing information needed to make difficult decisions in pregnancy outcome.

G45. Carrier Screening for Fragile X Through a Polymerase Chain Reaction-Based Method

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Introduction: Fragile X is the most common known cause of male related mental retardation. The disease causing gene, *FMR1*, is located on Xq27.3. A dynamic expansion of CGG repeats in the promoter region silences its expression in the affected individuals. Southern Blot analysis is the most common technique used to determine the expansion status of the CGG repeats in the *FMR1* gene. However, performing Southern Blot analysis is a relatively labor-intensive, time-consuming clinical technique. These limitations drive clinical laboratories to seek alternative techniques to replace the conventional Southern Blot in testing Fragile X. Here we present a PCR based high throughput method to determine the CGG repeat size in female patients planning pregnancy or in early pregnancy. **Methods:** The CGG repeat region is amplified using Abbott's *FMR1* PCR primers (set 1 and set 2), then sized through capillary electrophoresis. The individuals with CGG repeats range from normal to premutation can be accurately sized with primer set 1. The primer set 1 limits its power on the large allele due to the insufficient amplification of CGG repeats more than 230. Therefore, an individual with family history or presenting a large band on the agarose gel electrophoresis will be screened with primer set 2, which mainly detects the full mutation allele with higher sensitivity. **Results:** In our study, 2235 patients were screened using primer set 1 from 2009 through 2011. Among all the patients, 55 intermediate carriers (2.44%) and 10 premutation carriers (0.45%) were detected, respectively. In addition, a validation study was conducted with individuals that were normal, intermediate carriers, permutation carriers and full mutation carriers that were examined by using primer set 2. All the samples were read with the correct CGG repeat size information. This achieved the aim to test the accuracy and sensitivity of the primer set 2. **Conclusions:** In conclusion, we demonstrate a powerful PCR based screening method used for identifying the presence of intermediate mutation, premutation and full mutation in the *FMR1* gene.

G46. Molecular Follow-up of Newborn Biotinidase Screening in California Reveals Multiple Novel Mutations

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Introduction: Biotinidase deficiency is an autosomal recessive condition characterized by neurocutaneous symptoms including seizures, hypotonia, skin rash, alopecia, and developmental delay. Biotin supplementation initiated early in life prevents these sequelae. Therefore, newborn screening is offered in all states and many countries worldwide to identify potential patients and prevent clinical manifestations. Molecular analysis has enabled identification of mutations in the biotinidase (*BTD*) gene following enzyme analysis in serum, including the D444H mutation associated with partial biotinidase deficiency. Confirmatory molecular diagnosis is particularly useful in cases where sample mishandling may have lead to artifactually decreased biotinidase activity. **Methods:** Eighteen patients identified by newborn screening were enzymatically confirmed with profound (11) or partial (7) biotinidase deficiency. De-identified dried blood spots were received from the California Genetic Disease Screening Program for molecular analysis. *BTD* sequencing was performed using ABI Genetic Analyzer 3130. The assay targeted all four exons extending more than 20 bases into flanking introns and approximately 140 bases of 5'UTR. **Results:** *BTD* sequencing revealed two mutations in 10/11 newborns with profound deficiency. Seven of the 11 patients with profound deficiency had one novel mutation in conjunction with one severe known mutation, suggesting that the novel mutations are pathogenic. The known severe mutations identified include c.98_104del7insTCC, V62M, R157H, A171T, L176N, Y454C, Q456H and T532M. One patient with profound deficiency carried a novel missense mutation predicted to be pathogenic, a second mutation was not detected by our assay. Six of the seven patients with partial deficiency had one severe mutation together with the D444H (variant) allele, consistent with their enzymatic diagnosis. The seventh patient was homozygous for Y454C, a mutation previously reported *in cis* with R79C and *in trans* with R157H in a single patient; the enzymatic effects of Y454C homozygosity are unknown. Overall, we discovered eight novel mutations in our total of 18 samples (22% of alleles). Parent studies are required to determine the cis-trans relationships. **Conclusions:** The *BTD* sequencing assay identified two mutations in 17/18 samples with either partial or profound enzyme deficiency. The relatively large number of novel mutations detected in this cohort might reflect the ethnically diverse population in California. Sequence analysis is an excellent confirmatory test and can help differentiate between partial and profound biotinidase deficiency by the nature of mutations identified. Molecular follow up of enzymatic biotinidase assay is also likely to be beneficial for counseling purposes.

G47. Performance Characteristics of the eSensor® 2C19 Assay for the *CYP2C19* Genotyping

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Introduction: Recent studies have confirmed the importance of *CYP2C19* genotypes to outcomes in clopidogrel treated patients. The studies indicated that *CYP2C19*2* and other *CYP2C19* loss-of-function alleles play an important role in clopidogrel non-responsiveness and adverse clinical outcomes. Whereas, the *CYP2C19*17* has been associated with ultrarapid enzymatic activity and increased medication metabolism. The US Food and Drug Administration has added a box warning to the clopidogrel label suggesting that adjusting the dose or using alternative antiplatelet agents should be potentially implemented for high-risk individuals identified based on the *CYP2C19* genotype. The eSensor® 2C19 test for research use only (GenMark *CYP2C19* assay) (GenMark Diagnostics Inc, CA) identifies 12 nucleotide variants, including the ones relevant to clopidogrel. Here, we describe the performance characteristics of the GenMark *CYP2C19* assay. **Methods:** A reproducibility study was performed by two operators using the same kit lot. Eleven DNA control samples previously genotyped by the AmpliChip assay as *1, *2 and *3 were tested on 3 runs performed on three days. In a method comparison study, 63 DNA samples were genotyped using the GenMark *CYP2C19* assay and results compared to the expected genotype. These samples included 50 anonymized DNA patient samples previously extracted by the Qiagen EZ1 DNA Blood kit and genotyped by the Roche AmpliChip; 8 Coriell DNA samples characterized by the Roche Amplichip, AutoGenomics INFINITI, Laboratory Developed Test (LDT) RFLP, TaqMan LDT and Luminex; 3 ParagonDx controls and 2 proficiency testing samples. Eight anonymized DNA patient samples previously extracted by the Roche MagNA Pure LC DNA Isolation Kit were genotyped by the GenMark *CYP2C19* assay to evaluate the ability of different extraction methods to produce results. **Results:** Results from the reproducibility study were 100% correct when compared with the expected results. In a method comparison study, 100% concordance with the expected genotypes was obtained for 62 samples. One sample gave an invalid test result for two consecutive runs. When results for all the samples were compared to the AmpliChip

platform, 24/62 samples previously genotyped as *1 gave a *17 allele. This discrepancy is due to the fact that the AmpliChip assay does not interrogate for *17 allele. Genotype calls were obtained using DNA from both extraction methods. **Conclusions:** The GenMark *CYP2C19* assay provided good quality performance for the *CYP2C19* mutation panel that influence the metabolism of clopidogrel.

G48. AGG Genotyping Reclassifies Expansion Risk for Equivalently Sized Intermediate and Premutation Fragile X Alleles: Outcomes of a Multicenter Study of 469 Mother-Child Transmissions

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Introduction: Increased screening of pregnant women for their fragile X status has led to the identification of more intermediate and small premutation alleles whose stability is poorly understood. Earlier studies have suggested that the presence of AGGs interspersed within the repeat and the length of uninterrupted CGGs at the 3' end of the repeat may influence repeat expansion. Until now, the ability to determine AGG status in female samples has been technically prohibitive due to two X chromosomes. Thus, genetic counseling has been limited to interpretations of risk based solely on the number of CGG repeats. A combination of PCR methods has allowed us to examine the effect of AGG substructure on CGG repeat instability. **Methods:** Genomic DNA samples corresponding to 469 mother-child transmissions were obtained from 4 institutions following IRB approval. The number and allele location of interspersed AGG were established using the AmplideX™ *FMR1* CGG repeat primed PCR (RUO) and two novel research PCRs. **Results:** The number of AGG interruptions and the length of continuous CGG repeats fundamentally shifted the risk profile for CGG expansion. Between 50-54 repeats, the risk of expansion based on CGG repeats alone was 26%. However, in alleles of this size, the expansion risk was 100% without AGG, 28% with 1 AGG and only 11% with 2 AGG. Moreover, the magnitude of repeat expansion was larger for equivalently sized alleles that lacked AGG. The shortest unstable length was 25 uninterrupted CGGs (in a 45 CGG allele) and the smallest number of uninterrupted CGG repeats that expanded to a full mutation in a single transmission was 51 (of 61 total repeats). For females, the pure CGGs at the 3' end of the repeat region was the best predictor of the change in repeat length ($p<.0001$, $R2=0.21$) and explained twice the variance compared with total repeat length alone. **Conclusions:** This highly powered, large-scale AGG genotyping study provides the most definitive data to date on AGG interruptions. Either the number of AGGs or the 3' pure CGG repeat length more accurately estimates the risk for instability compared to the current standard of repeat size alone. AGG genotyping will thus allow risk estimates to be revised for repeat instability in newly identified intermediate and small premutation alleles.

G49. A Multiplexed Genotyping Test for *CYP2C19* Using VeraCode Beads on the BeadXpress System

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Introduction: Understanding the role of inter-individual genetic variability in drug response or disposition is a key step toward personalized medicine. The *CYP450 2C19* enzyme, which is encoded by the *CYP2C19* gene, plays a role in the metabolism of many drugs currently in clinical use. An individual's *CYP2C19* gene may contain genetic polymorphisms that affect the functional activity of the resulting CYP2C19 enzyme in that individual. This inter-individual variation in enzyme activity and resulting drug exposure can affect therapeutic response of drugs metabolized by CYP2C19. **Methods:** Here we describe the development of a new multiplexed assay, theVeraCode *CYP2C19* Genotyping Test, for the detection and genotyping of *CYP2C19* alleles on the BeadXpress® reader. The test is designed to detect the *CYP2C19* *2, *3, *4, *5, *6, *7, *8, and *17 alleles, and is intended to identify a subject's *CYP2C19* genotype using genomic DNA extracted from whole blood. The assay technology involves multiplex PCR followed by target specific extension (TSE) of the two alleles at the single nucleotide polymorphism (SNP) of interest. This process also incorporates a modified nucleotide that allows site specific labeling after hybridization of the amplified product to the VeraCode® beads. **Results:** The VeraCode *CYP2C19* Genotyping Test allows up to 96 samples to be genotyped within a 4-hour shift with less than 30 minutes of hands-on time from extracted DNA to genotype data. The assay had a call rate of >99% when tested on over 250 unrelated blood samples. Additional data generated with the Caucasian, Asian, and Yoruban HapMap samples (N=286) indicated excellent reproducibility (>99%) and accuracy (>99%) when compared to results obtained with alternative genotyping methods and bi-directional sequencing. For rare alleles that are present in the US population at a frequency of < 0.1%, synthetic, plasmid-based DNA samples were used to verify assay performance. **Conclusions:** The VeraCode *CYP2C19* Genotyping Test allows the genotyping of *CYP2C19* alleles in a simple, high throughput workflow with a short hands-on and total run time. This test is for Research Use Only.

G50. A Multiplexed Genotyping Test for *CYP2C9*/*VKORC1* Using VeraCode Beads on the BeadXpress System

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Introduction: Warfarin is an important anticoagulant that has large inter-individual variation in response and resulting maintenance dose requirements. A portion of this inter-individual variation in drug response is due to polymorphisms in the *CYP2C9* and *VKORC1* genes, whose gene products play a role in warfarin's pharmacokinetics and pharmacodynamics. Assessing a subject's *CYP2C9*/*VKORC1* genotype prior to initiating warfarin therapy may reduce the incidence of adverse events associated with warfarin therapy. **Methods:** Here we describe the development of a new multiplexed assay, the VeraCode *CYP2C9*/*VKORC1* Genotyping Test, for the detection and genotyping of *CYP2C9* (*2 and *3) and *VKORC1* (-1639G>A) variants on the BeadXpress® reader. The test is intended to identify a subject's *CYP2C9*/*VKORC1* genotype using genomic DNA extracted from whole blood. The assay technology involves multiplex PCR followed by target specific extension (TSE) of the two alleles at the single nucleotide polymorphism (SNP) of interest. This process also incorporates a modified nucleotide that allows site specific labeling after hybridization of the amplified product to the VeraCode® beads. **Results:** The VeraCode *CYP2C9*/*VKORC1* Genotyping Test allows up to 96 samples to be genotyped within a 4-hour shift with less than 30 minutes of hands-on time from extracted DNA to genotype data. The assay had a call rate of >99% when tested on over 250 unrelated blood samples. Additional data generated with the Caucasian, Asian, and Yoruban HapMap samples (N=286) indicated excellent reproducibility (>99%) and accuracy (>99%) when compared to results obtained with alternative genotyping methods and bi-directional sequencing. **Conclusions:** The VeraCode *CYP2C9*/*VKORC1* Genotyping Test allows the genotyping of *CYP2C9* and *VKORC1* genes in a simple, high throughput workflow with a short hands-on and total run time.
This test is for Research Use Only.

G51. Importance of Complete Genomic Analysis Using Conventional G-Banded Chromosome Analysis and SNP Microarray Analysis in Cases with Known Chromosome Abnormalities

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Introduction: Historically, G-banded chromosome analysis has been the gold standard for initial analysis of individuals with a complex clinical phenotype. Previous studies have clearly documented the utility of microarray analysis in the genomic study of these, suggesting that this may be more useful as a first-line test for these individuals. Array studies also are useful in further characterizing the genomic content of known chromosomal abnormalities. We compared results from cases with a known chromosome abnormality to the results obtained from microarray analysis to determine what percentage of chromosome abnormalities would be missed if an array-first approach were used. **Methods:** The Illumina Infinium assay was performed on approximately 3191 DNA samples using either the 370K, 610K or Omni1-Quad BeadChip Platform. B-allele frequency and Log2R ratio were analyzed using the Illumina GenomeStudio analysis software with DNA copy number changes prioritized using cnvPartition software. The majority of these cases had either a normal chromosome analysis result or had no previous chromosome analysis performed. However, 95 of the 3191 cases (3%) previously were known to have a visible chromosome abnormality by standard GTG-banding prior to array studies, which included suspected balanced rearrangements, known unbalanced rearrangements, mosaicism and aneuploidy. **Results:** Of the 3191 cases, 95 had a known chromosome abnormality, while 75 (78.9%) showed a clear benefit using SNP array analysis that more clearly characterized the abnormality present or showed no additional complexity to the previously known imbalance. However, 11 cases (11.6%) showed no evidence of a genomic imbalance suggesting that the rearrangement was truly balanced, and nine of the cases (9.5%) had a genomic imbalance that was an additional finding other than the suspected chromosomal abnormality or was in addition to the known aneuploidy suggesting a more complex genome is present than was anticipated. **Conclusions:** SNP microarray analysis is clearly a valuable tool in the analysis of cases with a complex clinical phenotype. However, if an array-first approach is utilized, there is still a clear benefit of performing standard G-banded chromosome analysis in these cases.

G52. Beyond Southern Blot Analysis: Fragile X Syndrome Case Studies and Analysis of New Sample Types Using *FMR1* Methylation PCR

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Introduction: Fragile X syndrome (FXS) is associated with the expansion of a CGG trinucleotide repeat in the 5'-untranslated region of the *FMR1* gene with consequent hypermethylation of CpG islands and gene silencing. While advances in *FMR1* PCR have led to the routine detection of full mutation alleles >200 CGG, molecular diagnosis of FXS still relies on Southern blot (SB) analysis to determine methylation status. However, SB analysis is extremely laborious, low throughput, and requires large quantities of genomic DNA. The quality of data can be ambiguous to interpret and novel sample types, such as hair follicle DNA that may be better correlated with clinical phenotype, are excluded. This study applies novel PCR technologies, including an optimized methylation PCR (mPCR-CE), to a series of fragile X case studies and including sample types of different cellular/germ layer origin. **Methods:** Genomic DNA from whole blood, hair follicle, and/or mouthwash samples were obtained from collaborators at Rush University Medical Center or the University of Zaragoza Medical School. DNA samples were processed using AmplideX™ CGG Repeat Primed (RP) PCR (RUO), and/or mPCR-CE research reagents. The results were compared with both SB analysis and cognitive and other clinical assessments performed by the clinical collaborators. **Results:** Cases representing both stable and unstable transmissions from parent to child, and the full range of methylation status in fully expanded *FMR1* alleles were evaluated. All mPCR-CE results from were highly correlated to phenotype and SB analysis. In addition, CGG RP PCR and mPCR-CE were performed to assess mesodermal (blood) and ectodermal (hair follicle and mouth cells) specimens from individuals within a family with two fragile X children. Repeat mosaicism and methylation status were dependent on the sample type with hair and mouth DNA showing greater correlation with low IQ than the whole blood samples. **Conclusions:** The evaluation of a novel PCR-only workflow across several fragile X case studies provided a comprehensive genetic profile including quantification of repeat size and methylation status in both mesodermal and ectodermal sample types. The research methods revealed both typical and atypical genotypes that were highly correlated to patient phenotypes. These results underscore the utility of PCR for routine CGG sizing and methylation assessment without SB analysis and may improve the understanding of fragile X genotype/phenotype correlations. In the future, this research may impact selection of patients most likely to respond to new treatments for FXS.

G53. Identification of New Mutations in the *KCNJ2* Gene in Patients with Andersen-Tawil Syndrome

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Introduction: Andersen-Tawil syndrome (ATS) is a rare autosomal dominant genetic disorder characterized by a triad of episodic flaccid muscle weakness, ventricular arrhythmias and prolonged QT interval, and anomalies such as low-set ears, ocular hypertelorism, small mandible, fifth-digit clinodactyly, syndactyly, short stature, and scoliosis. The clinical manifestations of ATS vary greatly among patients. *KCNJ2*, encoding the inward rectifier potassium channel 2 protein (Kir2.1), is the only gene known to be associated with ATS. The *KCNJ2* gene is located at 17q23, and its open reading frame is not interrupted by introns. The deduced protein sequence comprises 427 amino acids with two putative membrane-spanning regions connected by a pore-forming domain, and cytoplasmic N- and C-terminal domains. To date, more than 40 *KCNJ2* mutations have been reported to be responsible for ATS. **Methods:** We conducted *KCNJ2* mutation analysis in a cohort of 129 cases with suspected ATS referred to our center for genetic testing. Peripheral blood-derived genomic DNA was used to amplify the coding exon of the *KCNJ2* gene by PCR. The PCR products were purified and analyzed by bidirectional sequencing. Bioinformatics methods were employed to predict the functional consequence of the variants identified. **Results:** Mutational analysis showed that a total of 16 mutations in the *KCNJ2* gene were found in 34 patients. All of the mutations are missense mutations including 4 novel mutations not described previously, c.211G>T (D71Y), c.389C>T (T130M), c.578T>C (L193P) and c.653G>C (R218P), to our knowledge. To classify the four missense variants, computational analyses by SIFT and PolyPhen-2 revealed that three of the four missense variants (D71Y, L193P and R218P) might affect protein function of *KCNJ2*. Each of mutations T75M, T130M, G144S, T192I and R218Q was found in 2 patients, and R67W, R82W and G144A in 3, and R218W in 8. These reoccurring mutations may represent mutation hot-spots of *KCNJ2* in patients with ATS. **Conclusions:** Our study further expanded the mutational spectrum of the *KCNJ2* gene in patients with ATS. The data will be useful for the diagnosis and management of ATS patients. Functional studies of the four missense mutations identified are ongoing.

G54. Analytical Performance Characteristics of the Invader® *MTHFR* A1298C Test

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Introduction: The Invader® 5, 10-Methylenetetrahydrofolate Reductase (*MTHFR*) A1298C test is designed to use Invader Plus chemistry, a combination of PCR target amplification and Invader® signal amplification to detect and genotype a single point mutation (A to C at position 1298) of the human *MTHFR* gene in isolated genomic DNA obtained from whole blood. Studies were undertaken to determine the analytical accuracy compared to bi-direction sequencing, upper and lower limits of detection, interfering substances effects, and lot-to-lot reproducibility. **Methods:** Accuracy was determined by testing extracted DNA from 348 blood samples using the Invader *MTHFR* A1298C test and bi-directional sequencing and percent agreement determined. Upper and lower limits of detection were determined by testing 40 replicates of genomic DNA samples (wild-type and heterozygous genotypes) at concentrations of both 5 ng/µL and 80 ng/µL and at 10-fold extremes (e.g. 0.5 ng/µL and 800 ng/µL), prior to 1:20 dilution for the Invader® reaction, and the Invader® results compared to bi-directional sequencing. Heparin (1500 U/dL human whole blood), bilirubin (10mg/dL human whole blood), cholesterol (300mg/dL human whole blood), potassium EDTA (1.8 mg/mL human whole blood), hemoglobin (up to 0.2% in whole blood), and ethanol-based wash buffer (5% in DNA sample) were tested as potential interfering substances. Samples were also analyzed using the Invader® *MTHFR* A1298C test with three different lots of the reagents and observed agreement between all three lots and bi-directional DNA sequencing determined. **Results:** Observed agreement between Invader® *MTHFR* A1298C and bi-directional DNA sequencing was 99.71% (347/348 with 1/348 being an equivocal call), for upper and lower limits of detection determination at 5-80ng/µL concentrations agreement was 100% (80/80), and for samples tested at 10-fold extremes agreement was 67.5% (54/80) at 0.5 ng/µL (below the normal range of the assay) and 100% (80/80) at 800 ng/µL concentrations. All substances examined for potential interference had no impact on Invader® *MTHFR* A1298C performance. Observed agreement between three lots of the Invader® *MTHFR* A1298C test and bi-directional DNA sequencing was 100% (48/48). Across all genotypes tested, across all three (3) lots, the overall agreement with bi-directional sequencing was 100%. **Conclusions:** The Invader® *MTHFR* A1298C test is a sensitive, reproducible and highly accurate method for detecting and genotyping a single point mutation (A to C at position 1298) of the human *MTHFR* gene in isolated genomic DNA obtained from whole blood.

G55. A Familial Chromosome 2p Duplication in a Chimeric Infant with Nephrotic Syndrome

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Introduction: We describe here the clinical manifestations and chromosomal defects in a 4-month old infant who presented with nephrotic syndrome. The patient was born to non-consanguineous Hispanic parents with a non-significant maternal and pregnancy history but repeated pregnancy losses in maternal aunt. The infant has ambiguous genitalia with left cryptorchidism and left Ebstein anomaly with persistent proteinuria and slightly elevated serum AFP levels. Except for cryptorchidism and pulmonic stenosis, other characteristic features of the disorders of the RAS-MAPK pathway, Noonan (NS), Cardio-facio-cutaneous (CFC) or Costello Syndrome, were not present. We demonstrate an integrated laboratory work up and analysis that may help explain clinical findings in complicated cases such as ours. **Methods:** G-banded chromosomal study was performed in the patient's peripheral blood followed by array Comparative Genomic Hybridization (aCGH) with 1 Mb Hu BAC array. The same aCGH was done in the mother's sample. The patient underwent renal biopsy and right orchiectomy. Histologic, electron microscopic, and immunohistochemical examination with antibody to WT1 of the renal biopsy as well as histomorphologic examination of the excised testis were done. **Results:** Cytogenetic analysis showed 46,XY/46,XX and aCGH revealed a 110 kb (hg19: 39,227,561-39,337,844) duplication of chromosome 2p22.1 region in the infant. The same duplication was noted on aCGH of the mother. The duplicated region spans the *SOS1* gene on chromosome 2p21. The renal biopsy revealed diffuse global moderate mesangial hypercellularity with swollen proximal tubules. Immunohistochemistry with WT1 antibody revealed strong positive staining in podocyte nuclei. There was diffuse and complete effacement of the foot processes on electron microscopy. Histologic examination of the testis revealed ovotestis with chronic inflammation and fibrosis. **Conclusions:** We report a unique case of familial 2p22.1 duplication chimeric infant with ovotestis, Ebstein anomaly and nephrotic syndrome,

lacking typical features of NS-CFC-CS spectrum. From the scheme of laboratory examinations already performed and if mutation analyses of *NPHS1* and *NPHS2*, the most frequent causes of congenital nephrotic syndrome, proved to be negative, it is conceivable that the maternal origin of *SOS1* duplication is a pathologic copy number variant. Though *SOS1* missense mutations are second most common cause of NS and encode products that enhance RAS and ERK activation, inherited duplication of *SOS1* gene has not been reported to date. Besides the cytogenetic abnormality in our case, a defect in the RAS-MAPK pathway may possibly explain the phenotype in this case.

G56. Ongoing Experience Using a Prognostic Test for Prediction of Severe Curve Progression in Adolescent Idiopathic Scoliosis Patients

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Introduction: Adolescent Idiopathic Scoliosis (AIS) is a curvature of the spine greater than 10 degrees (Cobb angle) with no known cause. It affects 2% to 4% of the pediatric population. The majority of patients will not progress, but there is no reliable way to predict the expected clinical course. We have developed a DNA-based prognostic test (AIS-PT) to predict risk of progression to a severe curve. The test is designed to use at diagnosis of AIS. The AIS-PT identifies mild scoliosis patients at low risk of progressing to a severe curve (NPV > 99%). We report our experience over the first two and a half years of testing. **Methods:** The AIS-PT uses a DNA panel of 53 markers and current Cobb angle to assign a risk of progression score (1-200). Risk scores correlate with the patient's risk of progressing to a severe curve (>40° curve in an individual still growing). Saliva samples are collected, DNA extracted and genotypes determined using Taqman chemistry. The risk of progression score is calculated using a logistic regression algorithm. A physician survey regarding incorporation of the test into their practices has also been conducted. **Results:** Samples from 3181 patients have been submitted. 2791 patients were within indications for testing and were reported. The mean age of patients tested was 11.9 years for females and 12.6 years in males ($p < 0.177$). The mean Cobb angle is 18.7 degrees, 24% of girls tested were postmenarcheal and the most common initial Cobb value was 25 degrees. In the 1535 patients presenting with Cobb angles less than 20 degrees, 65.0% were identified as low risk and 0.5% were identified as high risk. In the 737 patients with Cobb angles less than 15 degrees, 82% were identified as low risk and no patients were identified as high risk. A total of 74 physicians responded to the survey. **Conclusions:** Initial use of AIS-PT testing has been directed toward older and higher acuity patients than most school screening series would predict as the expected populations. This is mostly likely because physicians using the test are seeing higher acuity patients. In patients most comparable to those expected from school screening populations (Cobb angle 10-15 degrees), the test performed similarly to validation populations where 75% of patients were identified as low risk. There were more out of indications samples submitted (12.3%) than expected indicating the need for additional education. Surgeons report that the test helps in making management decisions.

G57. A Multi-Center Reproducibility Study of the Invader® *MTHFR* C677T and Invader® *MTHFR* A1298C Tests

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Introduction: The Invader® 5, 10-Methylenetetrahydrofolate Reductase (*MTHFR*) C677T and Invader® *MTHFR* A1298C tests using Invader Plus chemistry, a combination of target amplification by PCR and Invader® signal amplification sequence detection by Invader® chemistry are designed to detect the *MTHFR* C677T and A1298C mutations on DNA isolated from human whole blood. A multi-center study was undertaken to determine the reproducibility of these assays. **Methods:** Testing took place at 3 sites with 6 operators (2 per site). The study was conducted in two main phases, proficiency phase (Phase 1) and an Invader® test performance phase (Phase 2). The proficiency phase ensured that each site and operator had the required expertise so that meaningful molecular test reproducibility measures could be calculated from the data generated from performance phase. Upon successful completion of Phase 1, Phase 2 testing took place, with samples for each of the two tests consisting of 9 human whole blood samples and 6 genotype-specific Invader controls, extracted using site-specific standard laboratory methods to obtain 5-80 ng/µl genomic DNA per sample. Samples included 3 representatives of each genotype, WT, HET and MUT and were extracted by each operator on each of 5 non-consecutive days. Extracted DNAs were tested in duplicate and genotype-specific controls were tested in singlicate. Reproducibility was assessed 1) within operator (within day); 2) between day (within operator); 3) between operators (within site); and 4) between sites. One-sided 95% confidence lower limits were calculated by the Wilson score method for each proportion of valid results as compared to independent bi-directional sequencing (SeqWright, Houston, TX). **Results:** There was 100% reproducibility for all 4 test conditions for both the *MTHFR* C677T and *MTHFR* A1298C tests. One-sided 95% confidence lower limits ranged from 99.0% (within operator/within day), to 99.5% for between day (within operator) and between operators (within site) for both tests. **Conclusions:** At three separate sites with six operators, all aspects examined demonstrated 100% reproducibility results for the Invader® *MTHFR* C677T test and the Invader® *MTHFR* A1298C test. The Invader® *MTHFR* C677T and Invader® A1298C tests demonstrated the reproducibility required for routinely detecting the *MTHFR* C677T and A1298C mutations in clinical blood samples.

G58. Is Self-Reported Race Sufficient in Molecular Genetics Clinical Testing Interpretations

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Introduction: The frequency of genetic variants can differ between races/ethnicities. These differences can alter interpretations of test results and change evaluation of risk. Typically, patients self report race/ethnicity. This information is used to interpret results where race/ethnicity risk differences are known. Another approach is to assume any race differences are small and not alter interpretations based on race. This tactic avoids the controversy and uncertainty of including race as an important factor of medicine. We have developed a DNA-based prognostic test to predict risk of progression to a severe curve in patients with Adolescent Idiopathic Scoliosis (AIS-PT). The test was originally validated in self-reported Caucasians and so we have limited the indicated use to that group, relying on self-reported race. In this study we sought to determine whether self-reported race was adequate to determine whether a patient was indicated for the test. **Methods:** The AIS-PT uses a DNA panel of 53 markers and current Cobb angle to assign a risk of progression score (1-200). Risk scores correlate with the patient's risk of progressing to a severe curve. Of the 53 markers, 19 show genetic differentiation between Caucasian, Asian and Black sub populations. Genotypes using these markers from 3816 self-reported Caucasian samples along with Hapmap 3 reference samples CEU (Caucasian), YRI (African) and CHB (Asian) were used to complete principle component analysis (PCA) to estimate the accuracy of self reported Caucasian ancestry. Samples submitted as Hispanic were added to the PCA analysis to look for the degree of Caucasian admixture in self-reported Hispanics. **Results:** In the PCA analysis the self-reporting status of Caucasians is highly accurate. 97.9% of self identified Caucasians fell within three SDs of the Caucasian cluster. 79 patients, however, fell outside this quadrant. 37 "Caucasian" samples should have been interpreted as Asian samples and 42 "Caucasian" samples should have been interpreted as African samples. When looking at self reported Hispanics the majority of the samples show significant admixture with Caucasians and should have received an interpretation. **Conclusions:** The data in our study shows self-reported race is usually accurate for Caucasians, however, a small percentage of patients should have received different interpretations. The majority of Hispanics could have been tested due to significant Caucasian admixture. In the future we plan to screen ethnicity using these 19 markers and for those falling outside the Caucasian cluster complete additional ancestry informative marker analysis so the most accurate interpretation can be given.

G59. Mutational Analysis of the *SOD1, FUS, TARDBP, ANG, OPTN, PON1, PON2*, and *PON3* Genes in Korean Patients with Amyotrophic Lateral Sclerosis (ALS)

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Introduction: Amyotrophic lateral sclerosis (ALS) is one of the most common adult-onset neurodegenerative diseases. Mutations in the *SOD1* are the most frequent genetic defects known to underlie ALS and to account for 20% of familial ALS (FALS) and 2% to 4% of apparently sporadic ALS (SALS). Recently, the cause of ALS has been attributed to mutations in several genes. However, there has been no report on the molecular genetic analysis for major ALS genes involving a large series in Korea. The aim of this study was to determine the mutation frequencies and spectrums of each major ALS gene in Korean ALS. **Methods:** Two hundred fifty-eight patients were included in the study; 9 FALS index cases and 249 SALS patients. We applied PCR and direct sequencing of all coding exons and flanking intronic sequences of the *SOD1, FUS, TARDBP, ANG, OPTN, PON1, PON2*, and *PON3* genes in all the patients. The sequences of the patients were compared with reference sequences to detect any sequence variations. **Results:** The overall frequency (FALS and SALS) of major ALS gene mutations was 8.1% (21/258); 88.9% (8/9) of FALS and 5.2% (13/249) of SALS. In FALS, 6 *SOD1* mutations were observed in seven patients and only one *FUS* mutation was found. In SALS, 3 *SOD1*, 4 *FUS* and each two *PON1, PON2,* and *PON3* mutations were found. Fourteen mutations were not previous reported. No mutation

was found in *ANG, TARDBP*, or *OPTN*. **Conclusions:** We determined the mutational spectrums of the major ALS genes in Korean ALS patients, which not only considerably higher frequency of *SOD1* mutations causing FALS, but also further highlight the importance of *FUS* and *PON*s. The frequency of *SOD1* gene mutations in Korean FALS (77.8%, 7/9) is much higher than those reported in the other ethnic groups but only one SALS case had *SOD1* gene mutation. Therefore, screening of *SOD1* gene is highly recommended in Korean FALS but other genes should be further studied in order to reveal the genetic background of Korean SALS.

G60. A Comprehensive Review of Genetics Sendout Testing

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Introduction: We have experienced, as perhaps others have, a steady increase in sendout testing requests for genetic diseases, notwithstanding active utilization review engagement with clinicians across the spectrum of clinical specialties over more than a decade. The fruits of the Human Genome Project and ongoing gene annotation efforts have contributed to this, yet our impression is the 'yield' on much of this testing is low. We wanted to confirm or refute that impression and assemble data for laboratorians and clinicians to evaluate circumstances in which sendout testing is most useful. **Methods:** Our plan is to review 5 consecutive years of experience 2006 – 2010. We have completed the review for 2009 of sendout genetic tests extracted from our laboratory's internal database. Permission was obtained from the UPMC Hospital QA Committee. Information extracted (or looked up) included test result, patient age, gender, and ordering physician. Data on current pricing of these tests was also gathered. **Results:** Test requests ranged from 1 to 14 for a given sample with a mean of ~2/sample. Some patients had tests on more than one sample. One clinician ordered 33% of all sendout tests. Of 2,576 discrete genetic sendout test results, 2,457 (95.4%) were for patients in whom there was no history of a known familial mutation. Of these, 301 (12.3%) gave 'positive' results that feel into ACMG category 1 and 2 variants. Variants of uncertain significance (VUS) were seen in (49) (2%) of tests. Of no surprise, 60 (50.4%) of 119 tests for known familial variants were positive. Gender was represented relatively equally (1,354 male vs. 1,223 female). Samples from children 0-10 and 11-20 years of age comprised 56% and 22%, respectively, of sendout tests requested; tests on patients older than 50 were 6% of total requests. A preponderance (68%) of tests were requested by medical geneticists (47%) and neurologists (21%), particularly in pediatrics. Total list price cost to the institution was $3,074,494, although discounts are available in some instances. **Conclusions:** This snapshot of data from one year's genetic sendout testing indicates a relatively low positive rate for results, particularly when one considers testing is 'confirmatory' for a number of patients who meet clinical criteria for a specific disease diagnosis. We are currently completing analysis for the other 4 years and plan to compile 'testing profiles' for discussion with specific specialty groups of physicians to identify criteria that enhance the use of genetic sendout testing in patient management.

G61. Analytical Performance Characteristics of the Invader® *MTHFR* C677T Test

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Introduction: The Invader® 5, 10-Methylenetetrahydrofolate Reductase (*MTHFR*) C677T test is designed to use Invader Plus chemistry, a combination of PCR target amplification and Invader® signal amplification to detect and genotype a single point mutation (C to T at position 677) of the human *MTHFR* gene in isolated genomic DNA obtained from whole blood. Studies were undertaken to determine the analytical accuracy compared to bi-direction sequencing, upper and lower limits of detection, interfering substances effects, and lot-to-lot reproducibility. **Methods:** Accuracy was determined by testing extracted DNA from 361 blood samples using the Invader *MTHFR* C677T test and bi-directional sequencing and percent agreement determined. Upper and lower limits of detection were determined by testing 40 replicates of genomic DNA samples (wild-type and heterozygous genotypes) at concentrations of both 5 ng/µL and 80 ng/µL and at 10-fold extremes (e.g. 0.5 ng/µL and 800 ng/µL), prior to 1:20 dilution for the Invader® reaction, and the Invader® results compared to bi-directional sequencing. Heparin (1500 U/dL human whole blood), bilirubin (10mg/dL human whole blood), cholesterol (300mg/dL human whole blood), potassium EDTA (1.8 mg/mL human whole blood), hemoglobin (up to 0.2% in whole blood), and ethanol-based wash buffer (5% in DNA sample) were tested as potential interfering substances. Samples were also analyzed using the Invader® *MTHFR* C677T test with three different lots of the reagents and observed agreement between all three lots and bi-directional DNA sequencing determined. **Results:** Observed agreement between Invader® *MTHFR* C677T and bi-directional DNA sequencing was 99.0% (359/361 with 2/361 being equivocal calls), for upper and lower limits of detection determination at 5-80ng/µL concentrations agreement was 100% (80/80), and for samples tested at 10-fold extremes agreement was 100% (80/80) at 0.5 ng/µL and at 800 ng/µL concentrations. All substances examined for potential interference had no impact on Invader® *MTHFR* C677T performance. Observed agreement between three lots of the Invader® *MTHFR* C677T test and bi-directional DNA sequencing was 100% (48/48). Across all genotypes tested, across all three (3) lots, the overall agreement with bi-directional sequencing was 100%. **Conclusions:** The Invader® *MTHFR* C677T test is a sensitive, reproducible and highly accurate method for detecting and genotyping a single point mutation (C to T at position 677) of the human *MTHFR* gene in isolated genomic DNA obtained from whole blood.

G62. Validation of a Laboratory-Developed Method Using Affymetrix® SNP 6.0 Arrays for High-Resolution SNP Analysis

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Introduction: SNP arrays can provide accurate genotyping data and kilobase-resolution detection of copy number variants (CNVs). We developed and validated a method using Affymetrix® SNP 6.0 chips for high-resolution SNP and CNV analysis. **Methods:** Genomic DNA was extracted from residual samples originally tested for various indications (36 blood samples tested by PCR for trisomy 13/18/21, X-Y aneuploidy, T-cell gene rearrangement, alpha-thalassemia, or *MTHFR* and *HFE* genotype; 10 amniotic fluid samples tested by PCR and GTG chromosome analysis for trisomy 13/18/21). DNA was digested with NspI and StyI, ligated to adaptors, and PCR-amplified. PCR product was purified using magnetic beads, fragmented, end–labeled, and hybridized to the chips, followed by staining and scanning. Data were analyzed using Affymetrix® GTC software. Obtained data were used to define data quality values and QC matrix. **Results:** All blood sample results were concordant with the original test, showing method accuracy. The mean call rate for clinical blood samples from >60 runs was 98.7%; contrast QC=2; and MAPD (median absolute pairwise difference)=0.28. The method demonstrated good reproducibility in inter- and intra-run studies: mean call rate >99%; contrast QC>2; and MAPD<0.29. One amniotic fluid sample failed PCR and was not tested on the chip; the remaining 9 generated results on SNP 6.0 chips concordant with the original tests, including correct gender calls. Analysis of additional samples is required to establish contrast QC and MAPD values for amniotic fluid. **Conclusions:** This method using SNP 6.0 chips showed high reproducibility and accuracy and can be performed within 3 days post-extraction.

G63. A Multi-Center Reproducibility Study of the Invader® FACTOR II (Prothrombin/G20210A) and Invader® Factor V (G1691A) Tests

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Introduction: The Invader® Factor II (Prothrombin/G20210A) and Invader® Factor V (G1691A) tests using Invader Plus chemistry, a combination of target amplification by PCR and Invader® signal amplification sequence detection by Invader® chemistry are designed to detect the Factor II (prothrombin/G20210A) and Factor V Leiden (G1691A) mutations on DNA isolated from human whole blood. A multi-center study was undertaken to determine the reproducibility of these assays. **Methods:** Testing took place at 3 sites with 6 operators (2 per site). The study was conducted in two main phases, proficiency phase (Phase 1) and an Invader® test performance phase (Phase 2). The purpose of the proficiency phase was to ensure that each site and operator had the required expertise in the fundamental methodologies so that meaningful molecular test reproducibility measures could be calculated from the data generated from the Invader® test performance phase. Upon successful completion of Phase 1, Phase 2 testing took place, with samples for each of the two tests consisting of 15 leukocyte-depleted human whole blood samples spiked with genotype-specific human cell lines were extracted using site-specific standard laboratory methods to obtain 5-80 ng/µl genomic DNA per sample. Samples included 3 representatives of each genotype, WT, HET and MUT and were extracted by each operator on each of 5 non-consecutive days. Extracted DNAs were tested in duplicate and genotype-specific controls were tested in singlicate. Reproducibility was assessed 1) within operator (within day); 2) between day (within operator); 3) between operators (within site); and 4) between sites. One-sided 95% confidence lower limits were calculated by the Wilson score method for each proportion of valid results as compared to independent bi-directional sequencing (SeqWright, Houston, TX). **Results:** There was 100% reproducibility for all 4 test conditions for both the Factor II and Factor V tests. One-sided 95% confidence lower limits ranged from 99.0% (within operator/within day) for both the Factor II (prothrombin/G20210A) and Factor V (G1691A) mutations, to 99.6% for between day

within operator) and between operators (within site) for both tests. **Conclusions:** At three separate sites with six operators, all aspects examined demonstrated 100% reproducibility results for the Invader® Factor II test and the Invader® Factor V test. The Invader® Factor II (Prothrombin/G20210A) and Invader® Factor V (G1691A) tests demonstrated the reproducibility required for routinely detecting the Factor II (Prothrombin/G20210A) and Factor V (G1691A) mutations.

G64. Next Generation Sequencing in Clinical Diagnostic Laboratories: Implementation of Quantitative and Qualitative Controls in Dual Genome Analysis

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Introduction: Advances in genomic technology have made sequencing of the entire human genome possible. The next generation sequencing (NGS) technology has been widely used in research laboratories. However its application to molecular diagnosis in clinical laboratories is still in its infancy due to the lack of guidelines from CAP or CLIA. The ability to sequence a group of target genes by NGS is particularly useful for molecular diagnosis of genetically heterogeneous clinical syndromes. Mitochondrial disorders are a group of complex diseases that can be caused by mutations in both nuclear and mitochondrial genomes. Current molecular diagnosis requires multiple different and complementary methods, including sequencing, qPCR, Southern blot or array CGH, for the detection and quantification of mutations. To develop a one-step comprehensive NGS for molecular diagnosis of mitochondrial disorders in a clinical setting with the implementation of proper quantitative and qualitative controls. **Methods:** A target gene selection followed by high throughput "deep" coverage NGS approach was validated with the indexed qualitative and quantitative controls analyzed along with each sample for quality assurance. **Results:** We demonstrated an average coverage of >500X for targeted nuclear genes and >5000X for each of the 16,569 bases of the mitochondrial genome. Nucleotide changes are correctly called with quantitative information. The limit of detection of a heteroplasmic change is calculated to be about 1.5%. Small and large insertion/deletions were correctly detected with clear breakpoints and percentage of heteroplasmy. **Conclusions:** This target gene enrichment deep sequencing approach provides a one-step comprehensive molecular analysis for patients with suspicion of mitochondrial diseases in a timely, accurate, and cost-effective manner suitable for clinical application. It allows simultaneous analysis of a set of nuclear genes targeted to mitochondria and the whole mitochondrial genome for point mutations and deletions with quantitative information. The inclusion of control samples assure the highest quality performance required in a clinical laboratory.

G65. Amyloid Precursor Protein (*APP*) Gene Study in Korean Alzheimer's Disease Patients

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Introduction: Alzheimer's disease (AD) is characterized by progressive cognitive decline. While the cause of AD has not been fully understood, genetics plays a role in the onset of the disease. Until now 4 genes are well known to be involved in AD; amyloid precursor protein (*APP*) gene, presenilin 1 (*PSEN1*) gene, presenilin 2 (*PSEN2*) gene, and e4 allele of apolipoprotein E (*APOE*) gene, which seems to be the most significant susceptible gene for sporadic and familial AD. In this study, we planned to screen *APP* mutations in codon 717 in Korean sporadic AD (SAD), minor cognitive impairment (MCI) and individuals with the normal cognitive function through PCR-RFLP. Simultaneously, sequencing analysis from codon 713 to codon 717 of exon 17 in *APP* genes was performed in SAD and MCI patients to investigate the presence of other point mutations. **Methods:** A total of 165 patients with complaints of the memory decline were included in this study performed at our hospital. In the outpatient clinic, patients underwent neurologic evaluations and a mini-mental state examination (MMSE). Thirty-two patients were diagnosed to probable SAD according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's disease and Related Disorder Association (NINCDSADRDA) criteria. Institutional Review Board approval and patient informed consent were obtained for this study. Total patients were divided into 3 groups according to the clinical diagnosis; SAD (n =32), minor cognitive impairment (MCI) (n =30) and normal cognitive function group (n =103). The exon 17 of *APP* gene was amplified using described specific primer; BclI (SIGMA, Saint Louis, USA) digests of these PCR products **Results:** A total of 165 patients with complaints of the memory decline were included in this study performed at our hospital. In the outpatient clinic, patients underwent neurologic evaluations and a mini-mental state examination (MMSE). Thirty-two patients were diagnosed to probable SAD according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's disease and Related Disorder Association (NINCDSADRDA) criteria. Institutional Review Board approval and patient informed consent were obtained for this study. Total patients were divided into 3 groups according to the clinical diagnosis; SAD (n =32), minor cognitive impairment (MCI) (n =30) and normal cognitive function group (n =103). **Conclusions:** The results of this study indicate that the presence of point mutation of *APP* is not a major cause of sporadic AD in the Korean population.

HEMATOPATHOLOGY

H01. Improved FISH Detection of Chromosomal Abnormalities Using CD138 Magnetic Microbeads Sorted Plasma Cells in Multiple Myeloma

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Introduction: Multiple myeloma (MM) is a plasma cell neoplasm. Karyotype and fluorescence in situ hybridization (FISH) provide valuable prognostic information. However, low percentage and slow growth of plasma cells in bone marrow samples have been the main obstacles in obtaining results reflecting the true lesions. Modified FISH protocols such as simultaneous FISH and immunohistochemistry staining for immunoglobulin light chain, and FISH following Giemsa staining for plasma cell capturing have been used for MM. We are validating a method of targeted FISH by plasma cell sorting and our preliminary results have showed a dramatic improvement in abnormality detection. **Methods:** Plasma cells were enriched by CD 138 magnetic microbeads for targeted FISH. A >90% of purity of plasma cells was reached as confirmed by May-Giemsa-Grünwald staining. For each bone marrow sample, FISH was performed on both sorted plasma cells and unsorted bone marrow cells for comparison. DNA probes for 5q31 (*EGR1*, D5S721-D5S23), 7q31 (D7S486, *CEP7*) and 14q32 (*IGH*) were used for validation. **Results:** Studies have been done on 14 cases and a comparison of the results has demonstrated a dramatic increase of abnormality detection when sorted plasma cells were used for targeted FISH. Chromosomal abnormalities were detected in 12 of the 14 cases (85.7%). In 2 cases (14%), regular FISH showed negative results with all the 3 probes while targeted FISH detected abnormalities in up to >86% of cells. Targeted FISH also revealed a much higher percentage of abnormal cells than the regular FISH did in the abnormal cases. For example, when FISH on unsorted samples detected additional copy of chromosome 5 in 7%, 8%, 12.5%, 20%, 25.5%, 48.5%, and 72.5% of cells, respectively, in 7 cases, targeted FISH detected this abnormality in 14%, 70.5%, 84.5%, 31%, 82%, 98%, and 100% of cells respectively. Similar results were also observed with the probes for chromosome 7 and 14q32. Interestingly, we observed abnormalities involving either chromosome 5 or 7 or both in up to 93.5% of cells without *IGH* rearrangement in 6 cases (6/14, 43%). **Conclusions:** We are in the process of validating targeted FISH by CD138 plasma cell sorting for routine clinical use. Our initial results have shown that this method can significantly improve abnormality detection for MM patients. IGH rearrangement has been considered as the primary event of MM. Our observation of chromosome abnormalities without IGH rearrangement in 6 of 14 cases suggests presence of complex genomic mechanisms of MM development and progression.

H02. Analysis of Clonal T-Cell Receptor Gene Rearrangements Utilizing Next Generation Sequencing

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Introduction: Over the past 20 years various methods for detection of gene rearrangements have been described with capillary electrophoresis now being the predominant method. Recently, the utilization of next-generation sequencing of PCR amplified products has been described in order to delineate the polyclonal repertoire of immunoglobulin and T-cell receptor gene rearrangements in genetic disorders, bone marrow reconstitution, and other clinical settings. The goal of the report is to demonstrate how next–generation sequencing could be used to detect clonal T-cell populations. **Methods:** Four cell lines (CEM, HSB2 JURKAT and MOLT4) derived from lymphoblastic leukemia and five patient samples were utilized for amplification with primers to the individual T-cell receptor gamma (*TRG*) genes and the families of T-cell receptor beta (TRB) genes. Peripheral blood was used as a polyclonal control. The sequencing was performed on an Illumina GIIA system by Adaptive TCR Technologies utilizing proprietary methods. The T-cell lines were chosen to illustrate a combination of known gene rearrangement sequences representing the different families of *TRG* variable and joining genes. Sanger sequencing of the *TRG* gene rearrangements was also performed in the four cell lines. **Results:** The number of sequences analyzed ranged from 250,000 to 6,000,000 sequences. *TRG* gene rearrangements including V2, V8, V9, and V10 were identified from the cell lines, but not the expected V11. The results of the patient samples demonstrate in two-dimensional and three-dimensional histograms the predominance of one T-cell receptor gene rearrangement over several others in the mixtures with polyclonal T cells. The 3D histogram analysis of peripheral blood demonstrates that some gene rearrangement families are more commonly used; similar to that seen in the normal distribution with capillary electrophoresis. Individual polyclonal sequences each comprised less than 0.1% of the total sequences in the

patient samples. Errors can be detected in sequences that each only comprises less than 0.01% of the total sequences. **Conclusions:** This work demonstrates the proof of concept for the use of next generation sequencing in order to identify clonal T-cell receptor gene rearrangements.

H03. Distinct Molecular Characteristics of *FLT3* Mutations Involving Multiple Internal Tandem Duplications

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Introduction: Internal tandem duplications (ITDs) of the juxtamembrane domain of the FMS-like tyrosine kinase 3 (*FLT3*) gene are associated with poor outcome in patients with acute myeloid leukemia (AML). Previous smaller studies have suggested possible prognostic significance of size and allelic burden of *FLT3*-ITD. A subgroup of *FLT3*-ITD mutations involves greater than 1 ITD, i.e. multiple ITDs (mITDs). The frequency of mITD and comparison with single ITD (sITD) has not been investigated. Here, we characterize a large number of patients with single and mITD mutations with a specific focus on size, allelic ratio and mutational profile. **Methods:** We reviewed the results of *FLT3* mutation testing in 10,351 bone marrow or peripheral blood specimens of patients with myeloid neoplasms (mostly AML). Exons 14 and 15 and the intervening intron of *FLT3* were amplified from DNA using fluorescent-based PCR with subsequent capillary electrophoresis to distinguish wild from mutant genotypes. The results of concurrent or subsequent molecular studies for mutations of *NPM1*, *RAS*, *CEBPA*, and *KIT* were included when available. Statistical analysis was performed using the Student's t-test and Fisher's exact test. **Results:** 571 (6%) samples showed a *FLT3*-ITD mutation. Of these, 362 (63%) showed 1 ITD and 209 (37%) showed >1 ITD with 121 (21%) cases showing 2 ITDs and 88 (15%) cases showing 3 ITDs. The size of the largest ITD in cases with mITDs was significantly longer (mean: 80, median: 66, range: 21-342) than cases with a sITD (mean: 54, median: 45, range: 3-240), p<0.005. Cases with mITDs showed higher percent allelic burden i.e. mutant/total (mean: 41, median: 44, range: 2-98) compared with cases with sITD (mean: 24, median: 17, range: 0.4-89), p<0.005. mITDs were less frequently associated with *NRAS* mutations (6/113, 5%) than sITD (22/122, 18%), p<0.005. There was no significant difference in the frequency of *NPM1*, *KRAS*, *CEBPA* and *KIT* mutations between the two groups. **Conclusions:** mITDs account for 37% of all *FLT3*-ITD mutations. mITD cases have longer ITD lengths, significantly higher allelic burden, and are less frequently associated with NRAS mutations. These results suggest that within *FLT3*-ITD mutations, mITDs represent a specific subgroup. With the availability of therapeutic *FLT3* and MAPK/RAS inhibitors, these results may have important clinical implications.

H04. Targeted Next Generation Sequencing of Clinically Significant Gene Mutations and Translocations in Leukemia

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Introduction: Leukemias are currently subclassified based on the presence of recurrent cytogenetic abnormalities and gene mutations. These molecular findings are the basis for risk-adapted therapy; however, such data are generally obtained by disparate, labor-intensive, and often low-resolution methods in the clinical laboratory. Using targeted next generation sequencing (NGS) we demonstrate that prognostically significant translocations and gene mutations, including SNPs and insertions/deletions (indels) can be identified simultaneously in multiplexed sequence data. **Methods:** We designed a capture panel consisting of 20 genes involved in leukemia prognosis including those involved as translocations and gene mutations. Capture probes were designed to 2X tile across both introns and exons of genes, and totaled ~1.0Mb in size. Genomic DNA was then isolated from five well-studied cell lines including OCI-AML3, MV4-11, K562, kasumi-1, and NB4, as well the non-enriched bone marrow of one patient with newly-diagnosed AML. Cell line DNA was sequenced in multiplex on an Illumina HiSeq with 2x101bp paired-end reads, while the patient sample was sequenced on a GAIIx with 2x60bp reads. Sequence data were then aligned to build 37 of the human genome using both bwa and Novoalign. SNPs were called with the GATK Unified Genotyper and common mutations filtered against dbSNP. Small and medium-sized indels were detected using Pindel, while translocations were identified using a combination of Breakdancer and Slope. All translocations were subsequently verified by PCR and Sanger sequencing. **Results:** Using our approach, we were able to identify all published mutations occurring in the five sequenced cell lines, including the 30bp *FLT3* IDT in MV4-11, *NPM1* insertion in OCI-AML3, and *KIT* D822K mutation in kasumi-1. Furthermore, we pinpointed DNA-level translocations in 3 of 3 cell lines with translocations covered by our capture panel. Similarly, we correctly identified the t(9;11) translocation in the patient-derived sample in addition to all commonly tested gene mutations. **Conclusions:** Using an analysis pipeline consisting of freely available software, we correctly identified all published gene mutations, including larger insertions, occurring in five cell lines, and simultaneously identified DNA-level translocations in 3 of 3 cases covered by the capture panel. We applied these same methods to patient-derived DNA with similar success. We found that by coupling paired and single end read analysis we were able to identify DNA-level translocations without false positive results by capturing only one translocation partner. We conclude that targeted NGS is a methodology well-suited to the detection of a full spectrum of clinically relevant mutations in hematologic malignancies.

H05. Identification of *MYC-IGH@* Translocation in Burkitt's Lymphoma by DNA-based Looped Ligation Assay (LOLA)

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Introduction: Burkitt's lymphoma (BL) harbors a hallmark translocation of *MYC* on chromosome 8q24 juxtaposed to one of the immunoglobulin heavy or light chain genes. Translocation breakpoints of *MYC* are scattered over 340 kb upstream of and within *MYC*, and the *IGH@* breakpoints in the switch α, γ, μ regions and the joining (JH) regions cover ~300 kb. Detection of this translocation at the DNA level is complicated. We have recently developed a ligation-based assay (LOLA) for long range haplotype mapping and detection of translocations. In this study, we employed LOLA to detect *MYC-IGH@* translocations in the DNA of BL cell lines. **Methods:** LOLA identifies linkage between widely separated genetic loci. Thirty oligonucleotide sets were prepared to cover a 200 kb span upstream of *MYC* (6 locations) and the switch (α, γ and μ) and JH regions and 50 kb upstream of JH region of *IGH@* (5 locations). DNA was isolated from BL cell lines (Raji, Akata, Rael, Daudi, and AG876). DNA (500 ng) and oligos were mixed and the ligation reaction was carried out at 60°C for 1 hr. PCR was then performed with primers complimentary to M13 tails on the outermost oligonucleotides. The diagnostic products were identified using capillary electrophoresis (ABI 3130) and GeneMapper software (ABI). **Results:** The LOLA produced a specific diagnostic peak in all samples tested. Different cell lines were discriminated by different combinations of oligos. Raji had breakpoints at exon 1 of *MYC* and the γ switch region of IGH whereas Akata and Rael had breakpoints at exon 1 and the μ switch region. The breakpoints for Daudi were 125 kb upstream of *MYC* and into the JH region. The AG876 breakpoints were located 150 kb upstream of *MYC* and at the μ switch region. Interestingly, the pattern of LOLA peaks in Raji and Akata indicates the presence of both derivative t(8;14) chromosomes. All findings matched either sequence data at the break points (Daudi, AG876) or mapping data (Raji, Akata) reported in the literature. This is the first report on the breakpoint for Rael. **Conclusions:** The LOLA assay is a powerful method for DNA-based detection of translocations where the locations of the breakpoints are not well known, such as in BL. It also can map translocations to a desired resolution and demonstrates the presence of derivative chromosomes. This strategy could be applied to identifying unknown breakpoints at the DNA level.

H06. Molecular and Cytogenetic Abnormalities in Acute Myeloid Leukemia - A Review and Description of Cases Studied at the Cytogenetics and Molecular Pathology Laboratory of a Brazilian Hospital

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Introduction: Cytogenetic abnormalities already well studied and new molecular markers are the basis for classification, prognosis and treatment in acute myeloid leukemia (AML). Particularly in AML with normal karyotype, *NPM1* and *FLT3* mutations are often associated with good and poor prognosis, respectively. In this study we have validated molecular tests for detection of *NPM1* and *FLT3* mutations and verified their frequency in AML patients sent to our laboratory. **Methods:** After approval of the institution ethical committee, a study was conducted from 03/2009 to 10/2010 in which 30 samples from AML patients were screened for *FLT3* and *NPM1* mutations. Blood samples were collected for PCR, cytogenetic (25 patients) and immunophenotyping studies. After DNA extraction, *NPM1* mutations and *FLT3*-ITD were detected through PCR reactions with a labeled primer and capillary electrophoresis analysis. *FLT3*-TKD mutation was detected through regular PCR and sequencing. Immunophenotyping studies were performed on a flow cytometer (EpicsXL, MCL) and G banding karyotypes were described according to ISCN 2009. AML stratification was performed according to Döhner et al, 2010. **Results:** Studying 30 samples from AML patients, we observed 33.3% frequency of *NPM1* mutation and an equal number for *FLT3*-ITD, which amounted to 50% and 40% in cases with normal karyotype. Eight percent of cases with normal karyotype and genotype *NPM1+/FLT3-* are included in the group of good prognosis AML. In 6 patients both mutations were found and we did not find *FLT3*-TKD mutation in our population. We did not observe the typical phenotype of AML with normal karyotype and mutated *NPM1* (HLA-DR and CD34 negative) in this series. **Conclusions:** In this small cohort, the frequency for *FLT3* and *NPM1* mutations was similar to the described by other groups. We did not detect any *FLT3*-TKD mutation,

which have shown a low prevalence in other Brazilian studies, when compared to American and European casuistic. Regarding immuphenotyping, we could not verify some literature data, such as a lower frequency of CD56 expression on *FLT3*+ patients or lack of CD34 and HLA-DR expression on *NPM1*+ patients. Finally, the study of *FLT3* and *NPM1* mutations in this cohort changed the prognostic classification in 8% of patients, from intermediate to low risk AML. Therefore, it is necessary to include molecular markers on the diagnostic workup of AML patients, in order to improve the classification, since this will impact directly on therapeutic decisions.

H07. Development of a Single Tube *TCRG* Assay

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Introduction: The human T cell receptor gamma (*TCRG*) gene locus has long been the target of PCR-based clonality testing, as rearrangement of these genes generate a clonal test result in greater than 90% of all people with T-ALL, and *TCRG* is a marker for a number of other lymphoproliferative disorders. However, analysis of *TCRG* test data has proved challenging, as false-positives can result from misinterpretation of minor peaks. Here we present data generated with an improved *TCRG* assay that detects the vast majority of *TCRG* rearrangements using a single, multiplex master mix. Master mix primers target all *TCRG* gamma variable regions and joining regions involved in gene rearrangements associated with lymphoid malignancies. The assay also generates a more comprehensive data set from patient samples, as some T-cell lymphoproliferative disorders involve V and J regions that would not be identified with a single Vγ(1-8) and Jγ1/2 primer set. Since all of the J region primers are labeled with the same fluorochrome the test also generates a more robust and easily interpretable Gaussian distribution. **Methods:** Amplification of similar-sized amplicons from all of the *TCRG* gene rearrangements should allow for identification of a quantitative threshold for a positive result and help to avoid false positive results. Twelve cell lines known to have clonal T-cell rearrangements were diluted into a tonsil DNA at a 5% level and were tested in parallel using the *TCRG* Gene Clonality Assay (BIOMED2 Tubes A and B) and the T Cell Receptor Gamma Gene Rearrangement 2.0 Assay (*TCRG*-6FAM, single tube). Amplification products were run on an ABI 3130xl capillary electrophoresis instrument. **Results:** Six of the cell lines tested with the *TCRG*-6FAM master mix generated two clonal products; testing the same six cell lines with the two BIOMED2 master mixes generated only one clonal product. Products generated with the new *TCRG*-6FAM master mix also exhibited a significantly higher fluorescent signal. Data indicate that this increased signal facilitates interpretation of ambiguous peaks. In addition, clonal products generated with the new *TCRG* master mix fall within the comparatively tighter, single contiguous size range of 159 base pairs to 207 base pairs, whereas the products generated using the two BIOMED2 master mixes fall into four different size ranges from 156 base pairs to 240 base pairs. **Conclusions:** Initial results show that this assay detects a wider range or rearrangements and provides a more clearly defined clonal peak within the polyclonal background.

H08. A Quantitative, Pyrosequencing-Based Assay to Detect *MPL* Mutations

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Introduction: Mutations in exon 10 of the myeloproliferative leukemia virus oncogene (*MPL*) are present in approximately 5% of patients with primary myelofibrosis (PMF) and 1% of patients with essential thrombocythemia (ET). *MPL* encodes a transmembrane receptor protein tyrosine kinase that acts as a receptor for thrombopoietin (TPO). The most common *MPL* mutations are the W515K and W515L mutations, which lead to augmented or constitutive activation of the *JAK*/*STAT* signaling pathway to TPO and contribute to the neoplastic phenotype. *MPL*-mutated patients typically demonstrate no evidence of *JAK2* mutations. Thus, *MPL* mutation testing represents an important component of the workup of patients suspected of having a myeloproliferative neoplasm. Here we describe a fast and easy quantitative pyrosequencing-based assay designed to detect *MPL* W515K and W515L mutations. **Methods:** Wild-type, W515K, and W515L plasmids were synthesized using standard amplification and site-directed mutagenesis reactions. One W515K, three W515L mutant patient specimens, and twenty *JAK2*-mutated specimens were subjected to granulocyte enrichment followed by PCR amplification of *MPL* in duplicate. Amplicons were subjected to Pyrosequencing on the Pyromark Q24 instrument using a nucleotide dispensation order designed to detect and quantitate *MPL* codon 515. Sensitivity and reproducibility experiments were carried out using diluted plasmid DNA. **Results:** The wild-type, W515K, and W515L plasmid specimens were amplified successfully and quantitative pyrosequencing indicated purity of >98%. One W515K and three W515L patient specimens yielded results similar to those from another reference laboratory, within 2%. Twenty *JAK2*-mutated specimens were determined to be wild type for *MPL* using our assay. Plasmid dilution experiments demonstrated sensitivity to 5% mutant in a background of wild type, and reproducibility experiments demonstrated that the 5% sensitivity was 100% reproducible. **Conclusions:** We have developed and validated a quantitative pyrosequencing-based assay designed to detect *MPL* mutations in PMF and ET. Our assay is robust, specific, sensitive, and reproducible and provides an important diagnostic aid for those patients who test negative for *JAK2* mutations.

H09. Genetic Polymorphisms and the Treatment Outcome in Standard-Risk Pediatric Acute Lymphoblastic Leukemia

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Introduction: Acute lymphoblastic leukemia (ALL) is the most common childhood cancer and accounting for 25% of all pediatric cancers in children. Only 50 years ago, the disease was uniformly fatal with an Overall Survival (OS) rate < 5%. Modern-day, multi-drug chemotherapy is associated with an overall survival rate over 80%. Standard-risk ALL comprises the majority of ALL with an overall survival approaching 90%. Despite this success, children who relapse from this disease accounts for the majority of pediatric cancer-related deaths. Treatment protocols have incorporated somatic but not host genetic features in the treatment regimens. The current study examined 12 genetic polymorphisms affecting the pharmacodynamics and pharmacokinetics of antileukemic drugs in an attempt to identify biologic markers related to the risk of disease relapse. **Methods:** Genomic DNA were extracted from the diagnostic bone marrow or peripheral blood specimens from 125 pediatric standard-risk ALL patients. Genetic polymorphism detection was carried out with either with TaqMan® genotyping techniques or conventional gel electrophoresis. Statistical analyses were performed with SAS/STAT software (SAS Institute Inc., NC) at the Colorado Biostatistics Consortium. **Results:** The GSTM1 null genotype was associated with a decreased risk of disease relapse (HR = 0.394, 95% CI = 0.127 - 1.224, P = 0.107). A combination analysis of the *GSTM1* and *GSTT1* genotypes revealed a stronger association between both the *GSTM1* and *GSTT1* normal genotype and an increased risk of leukemia relapse (HR = 2.73, 95% CI = 0.9 – 7.9, P = 0.063), compared with patients having either the *GSTM1* or *GSTT1* null genotype. A "drug exposure" model was used in this study. The risk of relapse in individual with a low or intermediate "drug exposure" genotype increased 2.4-fold (HR = 2.39, 95% CI = 0.8 – 6.9, P = 0.107) compared to the high "drug exposure" genotype. No significant associations with relapse were observed for the *CYP1A1, CYP2B6, CYP3A4, CYP3A5*, *MTHFR* C667T, *MTHFR* A1298G, or *TYMS* polymorphisms. **Conclusions:** The findings from this single institution study suggest that polymorphisms within genes of the *GST* superfamily may influence the treatment outcome in standard-risk ALL. They also point to the need for prospective, large multi-institutional studies to validate these findings prior to clinical implementation.

H10. Lyophilized Cell Controls for *BCR-ABL* Quantitative RT-PCR

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Introduction: Accurate detection and measurement of *BCR-ABL* transcripts by qRT-PCR is important for clinical management of patients with *BCR-ABL* positive CML and ALL. We report the development of controls for the common fusion transcripts, which can be used as full assay quality controls and validation **Methods:** These *BCR-ABL* controls are three panels of lyophilized cells that identify the b3a2, b2a2, and e1a2 *BCR-ABL* transcripts. Each panel consists of negative, 10^{-1} and 10^{-3} lyophilized cells. The negative cells are HL-60 cells that do not express any *BCR-ABL* transcripts. The 10^{-1} and 10^{-3} cells are *BCR-ABL* expressing cells diluted in HL-60 cells at 1:10 and 1:1000, respectively. Prior to lyophilization, cells were suspended in 2X PBS and filled into 3mL glass vials at approximate 1.0×10^{6} cells per vial. The freeze-dried products are capped in a desiccated nitrogen atmosphere. For each transcript, 3 batches of lyophilized cells were produced. The inter-batch variations were assessed for RNA yields using RNeasy Mini Kit. An in-house developed one-step qRT-PCR method was used to test these panels. The b3a2 panel was further evaluated using a laboratory developed assay for copy number of b3a2 and the endogenous gene BCR. **Results:** The average RNA yields per vial were 5.93±1.38 µg, 4.33±0.51 µg, and 4.77±1.21 µg, respectively, for b3a2, b2a2, and e1a2. Based on the one-step qRT-PCR method, the mean b3a2 Ct values and %CV were 21.95 (4.34%CV) for the 10^{-1} control and 29.03 (2.75%CV) for the 10^{-3} control. The mean b2a2 Ct values and %CV were 25.99 (3.53%CV) for the 10^{-1} control and 33.27 (1.89%CV) for the 10^{-3} control. The mean e1a2 Ct values and %CV were 29.30 (2.60%CV) for the 10^{-1} control and 36.67 (2.17%CV) for the 10^{-3} control. The ΔCt between the 10^{-1} and 10^{-3} controls were 7.08±0.37, 7.28±0.45, and 7.37±0.43 for b3a2, b2a2, and e1a2, respectively. Using a two-step qRT-PCR, the mean b3a2 copy number from 6 extractions was 2.72×10^{5} and 3.02×10^{3} for the 10^{-1} and 10^{-3} controls, respectively; the mean BCR copy number was 2.38×10^{5}, 4.07×10^{5} and 3.18×10^{5} for the negative, 10^{-1}, 10^{-3} controls, respectively; the

mean%b3a2/BCR was 74.88% and 1.04% for 10^{-1} and 10^{-3}, respectively; after applying the laboratory-specific IS conversion factor of 1.25, the mean%b3a2/BCR-IS was 93.6% and 1.30%. **Conclusions:** We have developed cell controls that can be used to monitor assay performance, including sample processing, for the *BCR-ABL* qRT-PCR assay.

H11. Testing for *WT1* Mutations in Acute Myeloid Leukemia

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Introduction: Cytogenetically normal acute myeloid leukemia (CN-AML) is a heterogeneous AML subgroup with intermediate-risk prognosis. Testing for mutations in several genes, including *NPM1*, *CEBPA*, *FLT3*, *DNMT3A*, and *WT1*, allows for further risk stratification of CN-AML patients. In addition, combinations of these gene mutations can lead to complex prognostic phenotypes. Here we describe our findings with a test for mutations in *WT1* that may be associated with a poor prognosis. **Methods:** Genomic DNA was isolated from residual whole blood and bone marrow specimens that underwent flow cytometric leukemia/lymphoma panel testing at ARUP Laboratories. *WT1* exons 3, 7, 8, and 9 were PCR amplified and sequenced. Results were correlated with flow cytometry phenotype and *NPM1* and *CEBPA* mutation status. No cytogenetic information was available. **Results:** Out of 88 total AML cases we identified 4 with a *WT1* exon 7 mutation. No mutations were found in exons 3, 8, and 9. In addition, 24 cases harbored the *WT1* exon 7 SNP rs16754, homozygous in 4 cases, which has been associated with a more favorable prognosis, especially in the *NPM1*/*FLT3* high-risk subgroup of CN-AML. Two cases harbored both the SNP and an exon 7 mutation. Two out of 4 cases with a *WT1* mutation also harbored favorable prognosis *CEBPA* double mutations and all *WT1* mutant cases were CD7 positive. **Conclusions:** We describe a test for identification of *WT1* mutations in AML. As previously reported, mutations were most frequently observed in the zinc finger region of the protein. The association of both poor (*WT1*) and favorable (*CEBPA*) prognostic markers confirms that accurate prognosis in CN-AML requires testing for multiple gene mutations.

H12. Sequencing the *PKLR* Gene after Enzymatic Detection of Red Cell Pyruvate Kinase (PK-R) Activity

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Introduction: Red cell pyruvate kinase (PK-R) deficiency is an autosomal recessive disease and a frequent cause of hereditary non-spherocytic hemolytic anemia. Mutations in the *PKLR* gene are associated with decreased PK-R enzyme activity and red cell hemolysis, putatively involving erythrocyte ATP depletion. Currently used enzymatic clinical assays for PK-R activity include a substantial inconclusive range, affecting diagnostic accuracy. Asymptomatic constitutional heterozygotes and some normal individuals have mildly decreased enzyme activity levels, whereas occasional symptomatic patients can show paradoxically normal to elevated PK-R activity, possibly due to reticulocyte or leukocyte interferences. Analysis of *PKLR* gene mutations may therefore be of value to unambiguously classify these patients. **Methods:** DNA was extracted from post-diagnostic patient blood samples analyzed for PK-R activity in the Mayo Clinic Metabolic Hematology laboratory. The *PKLR* gene was amplified using six primer sets and amplification products were directly sequenced using Sanger method (ABI 3130xl Genetic Analyzer) and analyzed for mutations using Sequencher software. Samples without mutations or with heterozygous mutation status were also screened by PCR for large deletions of exons 4 through 10 and exons 11 and 12. **Results:** Eighteen samples were evaluated for PK-R activity and sequenced for the *PKLR* gene. Two samples had decreased PK-R activities (<2.7 U/g Hb), one sample had an elevated activity of 20.1 U/g Hb, four were inconclusive (2.7 – 6.6 U/g Hb) and eleven samples had normal PK-R activity (6.7- 14.3 U/g Hb). Complex heterozygous mutations were found in both samples with PK-R activity <2.7. There was a single heterozygous mutation in one sample that had a low-normal value of 6.8 U/g Hb, and no other mutations or deletions were found in the remaining samples. **Conclusions:** Our sequencing-based strategy successfully identifies *PKLR* gene mutations in enzyme deficient patients. *PKLR* sequencing of samples with inconclusive or normal enzymatic results aids in the diagnosis of PK-R deficiency and can be of particular value when anomalous enzyme studies occur in clinically symptomatic patients. Although the gene mutation frequency is relatively high (0.6% to 6% in the general population) and asymptomatic heterozygous carriers frequently manifest mildly reduced enzymatic activity, our study demonstrates that many samples with mildly reduced enzymatic levels are not associated with mutations in the *PKLR* gene. In the setting of non-spherocytic hemolytic anemia, *PKLR* sequencing data is useful to distinguish those with clinically significant pyruvate kinase deficiency as the pyruvate kinase enzyme activity level can be decreased by unrelated causes.

H13. Molecular Analysis of *CEBPA* Gene Mutation in Cytogenetic Normal Acute Myeloid Leukemia (NK-AML) from Indian Population

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Introduction: The *CEBPA* is a transcription factor strongly implicated in myelopoiesis through control of proliferation and differentiation of myeloid progenitors. Mutation of *CEBPA* has been reported in ~10% to 15% of normal karyotype AML, and has been associated with good outcome. In the current study, we investigated the *CEBPA* mutations in AML patients with normal cytogenetics, and set out to explore its prevalence, distribution pattern and correlation with other molecular markers. **Methods:** Thirty-six AML subjects were investigated for *CEBPA* mutation by fluorescence-based multiplex PCR fragment analysis. All the data were analyzed using gene mapper software. The status of other molecular markers (*FLT3*, *NPM1*, *WT1* and *KIT*) was available as per our previous work. **Results:** Fragment analysis showed 7 *CEBPA* mutations in 4 (11.1%) of 36 AML patients. Mutations were detected throughout the entire coding region of *CEBPA* gene flanking the hot-spot regions of the TAD1, TAD2 (N-terminal) and BR, LZ domain (C-terminal) of the CEBPA protein. Three of the 7 *CEBPA* mutations were located in the N-terminal part of the protein, while remaining 4 of the 7 mutations were located in the C-terminal encoding the bZip domain of CEBPA protein. Among the four positive cases; 2 patients (#14 and #29) had mutations at N and C terminal regions. Of these, case #14 showed an insertion of +4bp, +5bp affecting the TAD1 and TAD2, and an insertion of +3bp probably affecting BR/LZ domain. The second case (#29) showed an insertion of +9bp and +3bp affecting TAD1 and BR/LZ domains respectively. The remaining 2 of 4 positive cases showed mutations only in C terminal region (#28 and #36). Notably, both of these cases revealed an insertion of +6bp each, most likely affecting the BR domain of the *CEBPA* protein. Assessment of *CEBPA* mutation and other molecular markers viz., *FLT3*, *NPM1*, and *WT1,* may permit normal karyotype AML to split into clinically distinct subgroups. Indeed we observed one case (#14) that had additional *FLT3* mutation. Another case (#29) showed simultaneous mutations in *CEBPA* and *WT1* gene, while interestingly none of the *CEBPA* mutant cases showed any association with *NPM1* and *KIT* mutation. **Conclusions:** The prevalence of *CEBPA* mutations observed in the current study is comparable to worldwide incidence (10% to 15%). Available literature suggests *CEBPA* mutation to be associated with good clinical outcome (in absence of *FLT3* mutation); hence its inclusion in diagnostic evaluations of AML may allow assignment of patients to better-defined risk categories.

H14. Promoter Methylation of *CDKN2A*, *MGMT*, and *CDH1* in Multiple Myeloma

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Introduction: Aberrant DNA methylation is involved in the initiation and progression of carcinogenesis and includes hypermethylation of CpG islands of tumor suppressor gene promoters. Cyclin-dependent kinase 2A (*CDKN2A*), O6-methylguanine DNA methyltransferase (*MGMT*), and E-cadherin (*CDH1*) are known to be hypermethylated in various neoplasms. We investigated the methylation status of these genes using pyrosequencing in multiple myeloma (MM). **Methods:** Three cell lines, ARH77, RPMI8226, and MC/CAR, and bone marrow aspirates from 10 patients with MM were analyzed by pyrosequencing. The methylation index (MtI) of each gene promoter was calculated as the average value of methylated cytosine [(mC)/(mC+C)]. Hypermethylation was defined as MtIs of *CDKN2A* >0.047, *MGMT* >0.053, and *CDH1* >0.081. **Results:** CpG island hypermethylation of promoters of *CDKN2A*, *MGMT*, and *CDH1* were found in all the MM cell lines. Mean MtI of *CDKN2A*, *MGMT*, and *CDH1* in cell lineswere 0.937, 0.250, and 0.576, respectively. Hypermethylation of *CDKN2A*, *MGMT*, and *CDH1* were detected in 80% (8/10), 56% (5/9), and 89% (8/9) of the MM patients, respectively. Mean MtIs of *CDKN2A*, *MGMT*, and *CDH1* in the MM patients were 0.108, 0.093, and 0.243, respectively, which were significantly higher than control group (P<0.05). **Conclusions:** Our results suggest that hypermethylations of tumor suppressor genes (*CDKN2A*, *MGMT*, and *CDH1*) are frequent events in MM, which may play an important role in the progression of carcinogenesis. Pyrosequencing is useful for detection of methylation and it offers quantitative data and high throughput.

H15. MicroRNA Profiles in Chronic Lymphocytic Leukemia

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Introduction: miRNAs have been shown to modulate hematopoietic lineage differentiation and play important gene-regulatory roles. Several miRNAs are known to be either up- or down-regulated in chronic lymphocytic leukemia (CLL). In this study we evaluated an eight miRNA profile in patients with CLL. **Methods:** miRNA was isolated

from B-cell enriched (RosetteSep Human B Cell Enrichment Cocktail, StemCell Technologies, Vancouver, BC, Canada) peripheral blood from 60 individual CLL samples that was passed thru a ficoll Hypaque gradient. The patients' ages ranged from 47 to 92 years and 25 of these were treated previously while 35 were untreated. Total RNA was extracted using the mirVana™ miRNA Isolation *KIT* (Ambion). Expression of miR15a, miR16-1, miR29a, miR181a, miR155, miR21, miR223, miR29c and U47 (an endogenous control) in each sample was determined using TaqMan® MicroRNA Assays (Applied Biosytems). In addition, expression of these miRNAs was determined in B cells from normal samples. The ΔCT of the normal (CTnormal - CTendogenous control) and CLL samples (CTCLL sample - CTendogneous control) were calculated, and relative comparison of normal and CLL samples was made using the ΔΔCTmethod (ΔCTCLL sample - ΔCTnormal). **Results:** In this study, we found that there was no change in expression patterns for miRNAs 181a and 155 between the treated and untreated groups. miR15a and miR16-1 expression was similar to baseline controls in the treated population but down-regulated in 28% of the untreated samples. miR29a was down-regulated in both patient populations. miR21 and miR29c were up-regulated in all of the treated CLL patients but down-regulated in 20% and 17%, respectively, of the untreated samples. While miR223 was down-regulated in both patient populations, there was a 6 log decrease in the untreated samples versus the treated samples. All values were normalized to the U47 control miRNA and a significant result was considered as a change greater than 1 log. **Conclusions:** Our results show that miRNA expression levels in CLL patients differ significantly between treated and untreated patients, as well as from that of normal patients. These results suggest that one role for miRNA profiling may be in determining the prognosis and potential outcomes in treated CLL patients. Further studies are needed to confirm these findings.

H16. Multi-Site Evaluation of a Multiplex Assay for the Rapid Detection of Leukemia-Associated Fusion Transcripts

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Introduction: Selection of optimal treatment approaches for leukemia patients entails accurate risk-based classification and analysis of multiple recurring chromosomal abnormalities. At the molecular level, analysis of the corresponding fusion transcripts confirms the initial diagnosis, provides additional prognostic information, and pinpoints targets suitable for subsequent disease monitoring. Rapid molecular methods are required to complement standard cytogenetic techniques and speed-up the workup of leukemia patients. The objective of this study was to evaluate the performance of a multiplex molecular assay for the simultaneous detection of 12 leukemia fusion transcripts and to compare to standard cytogenetic methods. **Methods:** The Signature® LTx RUO (Research Use Only) was evaluated at 3 sites using archived total RNA isolated from whole blood or bone marrow. Total RNA (100-400 ng) was reverse transcribed into cDNA and amplified by multiplex PCR using target-specific, biotin-modified primers. GAPDH transcripts were co-amplified in each sample and concurrently analyzed to serve as endogenous internal controls. The PCR products were then sorted on a liquid bead array containing oligonucleotide probes specific for each marker and detected using the Luminex® 100 or 200 Systems. **Results:** A total of 198 residual RNA samples were successfully tested at 3 sites. Specific detection of *BCR-ABL*1 e13a2, e14a2, or e1a2, *TCF3-PBX1*, *ETV6-RUNX1*, *MLL-AF4* e9e5 or e10e4, *PML-RARA* bcr1 or bcr3, *CBFB-MYH11* type A or D, and *RUNX1-RUNX1T1* was confirmed in 105 positive samples. There was >98% agreement with historical karyotype and/or FISH data. The assay helped resolve complex cytogenetic cases and positively identified the expected fusion transcript in RNA samples from patients with low blast count or at relapse. Additional analytical experiments with cell line RNAs and synthetic transcripts confirmed assay specificity, established a preliminary sensitivity of at least 1%, and showed that the panel content can be increased to include additional rare variants and other fusion transcripts. **Conclusions:** The assay is compatible with representative RNA samples extracted from various lymphoid and myeloid malignancies, including AML, CML, ALL, MDS and MPN. The assay has the advantage of typing individual fusion transcripts that can facilitate follow-up analyses and disease monitoring. It can also streamline laboratory operations with results generated in less than 5 hours and a multiplex format compatible with panel expansion. Overall, the assay is a sensitive and specific molecular research tool attuned to the clinical laboratory workflow and complements standard cytogenetic methods.

H17. *KIT* Mutation Is Rare in Acute Myeloid Leukemias, Almost Always Involves Exon 17, and Occurs Predominantly in AML with t(8;21)(q22;q22)

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Introduction: Activating *KIT* mutations play a role in leukemogenesis and are most frequently reported in core binding factor acute myeloid leukemias (AML). We review our experience with *KIT* mutation frequency in a large cohort of AML patients, and correlate *KIT* mutational status with prognosis. **Methods:** We searched our database for AML cases that were assessed for *KIT* mutations from 2002-2010. Mutational analysis of *KIT* exons 8 and 17 was performed by PCR and sequencing. Clinical and laboratory data were obtained from the medical records. **Results:** 16 of 1150 (1.4%) AML cases had *KIT* mutation including 10 women and 6 men with a median age of 44 years (range, 19-65). *KIT*-mutated cases were classified using the WHO scheme as: AML with t(8;21)(q22;q22) (n=11), AML with inv(16)(p13.1q22)/t(16;16)(p13.1;q22) (n=2), AML without maturation (n=2), and AML with myelodysplasia-related changes (n=1). All patients had thrombocytopenia, 14 had anemia, 6 had leukocytosis, and 6 had leukopenia. The median blast count in bone marrow was 74% (range, 24% to 96%). Flow cytometric analysis showed CD19 and CD56 expression in 7 and 10 cases of AML with t(8;21)(q22;q22), respectively. Four patients had t(8;21)(q22;q22) as a sole aberration; others had more than one abnormality including +4 in 3 and +8 in 2 patients. *KIT* mutations were identified in exon 17 in 14 patients (87%, 13 codon 816, 1 codon 822) and exon 8 in 2 (1 codon 413, 1 codon 421). The mutation L421I has not been reported previously. *NRAS* mutation was detected in 2 patients; none had *FLT3* mutation. All patients received chemotherapy, and 3 underwent bone marrow transplantation. With a median follow-up of 10.5 months (range, 3-53), 8 patients died of disease, 6 remained in clinical remission, and 2 had persistent AML. Compared with patients with *KIT*-negative AML with t(8;21)(q22;q22) seen during the same interval (n=29), *KIT*-mutated patients were more frequently women, and had a higher bone marrow blast count and shorter overall survival (p<0.05). **Conclusions:** *KIT* mutations in AML are rare, and occur almost exclusively in core binding factor AMLs, particularly AML with t(8;21). *KIT* mutations almost always involve exon 17, and are associated with shorter overall survival. Our data suggest that routine screening of all AML patients for *KIT* mutation is not necessary, nor is routine analysis of exon 8 indicated. *KIT* mutation analysis can be focused on exon 17 and be performed in patients with core binding factor AML, particularly patients with AML with t(8;21).

H18. *DNMT3A* Mutations in Acute Myeloid Leukemia and Myelodysplastic Syndrome

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Introduction: Somatic mutations in the DNA methyl transferase 3A gene (*DNMT3A*, 2p23) were discovered in 20 to 22% of *de novo* AML and recently in MDS and MPN. The most common *DNMT3A* mutation in exon 23 at codon R882 was found in more than 60% of AML cases in the initial report. Most of these mutations were located in one the three functional domains of DNMT3A protein: methyltransferase (MTase, 634-907 aa) encoded by exons 16 through 23, proline-tryptophan-proline (PWWP, 289-362 aa) and Zinc finger (ZNF, 528-605 aa) encoded by exons 8-9 and exons 14-15, respectively. Mutations in *DNMT3A* provide new insights into pathogenesis of myeloid malignancies and could lead to new therapeutic possibilities. In addition, *DNMT3A* mutations were associated with poor outcome. **Methods:** We studied a series of 71 AML (both *de novo* and secondary) and 31 MDS cases for mutations in exons 14, 15, 17, 18, 19, and 23 of *DNMT3A* in a. PCR amplification of the 6 exons was performed in 5 separate reactions (exons 18 and 19 were amplified together). PCR products were purified and sequenced by the Sanger method in forward direction. All positive cases were re-amplified and bidirectionally sequenced. **Results:** We identified *DNMT3A* mutations in 11 AML cases (11/71=15.5%) and 2 MDS cases (2/31= 6.5%). A total of 15 *DNMT3A* mutations were found in the 13 cases (9 males and 4 females). Two AML cases had two mutations each (p.598X/p.V657M; p.R882H/p.V690G). Eight mutations were located in exon 23 at codon 882 (6 R882H, 2 R882C), 3 in exon 15, 3 in exon 17 and one in exon 19. Six mutations were not reported previously: one frameshift mutation in exon 15 p.E561fs650X that adds 90 amino acids before a stop codon, one in frame deletion in exon 17 p.E667_I770delEDSI, and two missense mutations: p.R596Q, p.V657M, p.V690G, and p.F734L. No mutations were found in exons 14 and 18. **Conclusions:** Our results confirm the presence of *DNMT3A* mutations in a significant number of AML cases and in a lower frequency in MDS. Testing for *DNMT3A* mutations in AML and MDS patients may be of prognostic value and could be used as a marker to detect minimal residual disease.

H19. Protein-Tyrosine Phosphatase, Nonreceptor-Type 6 (*SHP1*) Expression Loss as an Alternate Drug Resistance Mechanism in Chronic Myelogenous Leukemia

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Introduction: Chronic myelogenous leukemia (CML) patients can be refractory to imatinib mesylate therapy targeting the *BCR-ABL* kinase. Resistance in the absence of detectable abl kinase mutations suggests the presence of alternative mechanisms of resistance. The protein-tyrosine phosphatase, nonreceptor-type 6 (*PTPN6, SHP1*) down-regulates (dephosphorylates) signal transduction factors required for cell proliferation. The purpose of this study is to investigate the role of *SHP1* in CML patients with and without detectable abl kinase mutations. We hypothesize that decreased expression of *SHP1* is an alternative mediator of imatinib resistance. **Methods:** Sensitivity to imatinib was defined as a three log decrease in BCRabl/abl transcript levels in12 months or less. Transcript numbers were quantified using qPCR standard curves made with synthetic oligomers. The ratio of *SHP1* transcripts/abl transcripts (*SHP1/abl*) was measured in RNA from 53 imatinib-sensitive and 47 imatinib-resistant patients with CML and ten normal controls. **Results:** Average *SHP1/abl* was similar in normal cells and CML (p=0.747). The average *SHP1/abl* ratio was lower in imatinib-resistant CML cases (18.6), than in imatinib-sensitive cases (29.2; p=0.172). *SHP1* transcript levels equal to or less than *abl* transcript levels (*SHP1/abl* <1.00) were significantly associated with resistance to imatinib (Chi-Square p=0.020). Taking account of *abl* kinase mutations, T315I, M351T, E255V and E255K, the average *SHP1/abl* ratio was highest in five sensitive cases found to have mutations, 42.7 vs 27.7 in sensitive cases (n=48) without *abl* kinase mutations. Lower levels of *SHP1* were observed in seven resistant cases with mutations (22.2). The average *SHP1*/abl ratio was lowest in imatinib-resistant cases without detectable abl kinase mutations (18.0; n=40). **Conclusions:** The results are consistent with *SHP1* loss contributing to imatinib resistance in CML. Negative regulation of signal transduction factors, including *src* kinases, by *SHP1* phosphatase would suggest a mechanism for the activity of *src* kinase inhibitors such as dasatinib or dual agents such as bafetinib. These agents may counteract loss of *SHP1* activity in imatinib-resistant cases of CML or ALL, especially those without detectable *abl* kinase mutations.

H20. Array Cytogenomics as a Diagnostic Aid for Acute Myeloid Leukemia: A Comparison of Four Different Platforms

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Introduction: Array-based cytogenomic platforms have become mainstream diagnostic tools for constitutional chromosomal abnormalities not detectable by traditional cytogenetics. However, the chromosomal complexities seen in human cancer have delayed the implementation of this technology for personalized tumor analysis. As a first step towards validating array-based cytogenomics for cancer specimens, we selected eight cases of acute myeloid leukemia (AML) with defined cytogenetics and assayed them using four competing array platforms from Illumina, Affymetrix, Nimblegen-Roche and Agilent. Here we present the results of our comparative analysis, which demonstrate the relative strengths and weaknesses of the technologies. **Methods:** Eight cases of AML representing a spectrum of cytogenetic findings were selected for molecular analysis following routine cytogenetic analysis of bone marrow aspirates. DNA was isolated pre-culture (Puregene, Gentra) and submitted for array analysis at each company's laboratory. DNA was then processed on the four array platforms, and the resulting data was returned for our review. Data analysis, including aberrant calls, was also provided by several of the participating companies to allow for comparison of our analysis pipeline with the company's analysis software. **Results:** Three of the platforms tested included both copy number and genotyping probes (Illumina, Affymetrix and Agilent), while the Nimblegen platform analyzed copy number only. Cytogenetically visible abnormalities present in a high percentage of cells were generally detectable by all platforms. Detection of abnormalities present at low levels was more complicated, with each platform having different detection limits. Inclusion of SNP probes allowed for increased sensitivity to low level events, and was crucial for detecting copy neutral loss of heterozygosity not detectable by cytogenetics. Probe density for both copy number and genotyping probes was also found to be important for accurately determining the size of affected regions. Sensitivity to detecting the percentage of cells affected by each abnormality also proved to be important in determining the clonal diversity of the tumor. **Conclusions:** Analysis of our samples on all four platforms allowed us to assess the strengths and weaknesses of each of the underlining technologies. Given the types of aberrations found in malignancies and issues related to clonality, key aspects required for successful incorporation of arrays into cancer cytogenetics include: the power to detect copy neutral LOH and aberrations present in a low percentage of cells, and the ability to accurately quantify the percentage of cells affected by each abnormality. Each platform analyzed has overlapping abilities, with no single platform performing best in all areas.

H21. *TP53* DNA-Binding Motif Mutation Is Found in High-Risk, Untreated Chronic Lymphocytic Leukemia Patients with Chromosome 17p Deletion

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Introduction: Interstitial deletion of chromosome 17p (17p-) detected by fluorescence in situ hybridization (FISH) and p53 mutations are unfavorable prognostic markers in chronic lymphocytic leukemia (CLL) and are associated with significantly poorer response to chemoimmunotherapy and decreased overall survival. We applied SNP-based whole genome copy number variation (CNV) analysis to characterize CLL patients with 17p-. DNA sequencing was used to identify mutations in the *TP53* gene. **Methods:** We performed CNV analysis and *TP53* sequencing on 6 patients with early-intermediate stage, untreated CLL who had high risk for disease progression based on molecular and immunophenotypic markers. All six patients had 17p- and 13q- detected by FISH. CLL cells and normal cells were separated from patient peripheral blood by immunomagnetic beads. CNV analysis was performed on purified genomic DNA from CLL and normal cells for each patient to distinguish acquired copy number changes in malignant cells from polymorphic CNVs in the human genome. The Illumina human 660w-quad beadchip, a SNP-based microarray, was used for CNV analysis. Data was analyzed by CNV partition and PennCNV software. *TP53* mutation analysis was performed by dideoxy sequencing of PCR products amplified from *TP53* exons 4-9. **Results:** CNV analysis detected 17p- in 6 out of 6 patients with 17p- by FISH. One sample had 17p- in 20% of nuclei by FISH, which is near the current detection level of SNP-based copy number analysis (estimated at 15% to 20%). Four patients had hemizygous deletions covering the entire p arm of chromosome 17. Two patients had hemizygous interstitial deletions of 17p that spanned 17 Mb and 580 Kb respectively. All 17p deletions included the region of *TP53*. Additionally, patients with 17p- had numerous acquired copy number aberrations (CNAs) in the CLL genome located on chromosomes 1, 2, 3, 4, 9, 10, 13, 15, 18, and 20. These CNAs included hemizygous deletions, homozygous deletions, duplications, and uniparental disomy. Four of the six 17p- patients had *TP53* mutations located in the p53 DNA-binding motifs (DBMs), specifically between amino acids 109 and 286. **Conclusions:** SNP-based CNV analysis enables detailed characterization of complex copy number aberrations in the CLL genome, including large and small interstitial deletions of chromosome 17p. The majority of our CLL patients with 17p- also have *TP53* mutations involving the DBMs, which have been associated with especially poor prognosis. Analysis of *TP53* mutation status complements SNP-based CNV evaluation. *TP53* screening is essential in CLL patients requiring treatment for progressive disease.

H22. Detection of Minor Clones with Internal Tandem Duplication Mutations of *FLT3* Gene in Acute Myelogenous Leukemia Using Delta-PCR

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Introduction: Internal tandem duplication (ITD) mutations of the *FLT3* gene have been associated with poor prognosis in acute myelogenous leukemia (AML). Detection of minor clones or minimal residual leukemia clones with ITD mutations may be essential. In clinical diagnostic laboratories, ITD mutations are usually detected using PCR followed by capillary electrophoresis with an analytic sensitivity of 5% to 10% blasts. Since the potential amplicon size varies greatly and multiple mutations may be present, PCR detection of ITD mutations using capillary electrophoresis may be challenging, particularly when the peak height is low. **Methods:** In our previous study, we developed a triple-primer strategy, delta-PCR, to ensure PCR specificity and to improve sensitivity. The primer design is analogous to semi-nested PCR, but all three primers are used simultaneously in a single-step reaction. The internal primer functions as a confirmatory "probe", since it was designed at a defined distance (the delta) from the external primer. A pair of amplicons differing in size by the delta indicates an ITD mutation. In the current study, delta-PCR was used to detect ITD mutations with a sensitivity of 0.1% leukemic cells. 109 cases with newly diagnosed or relapsed AML were analyzed, including serial samples from 3 cases. **Results:** Delta-PCR was able to detect single or multiple ITD mutations with an allele burden (peak height ratio of mutant allele to wild-type allele) of the dominant clone ranging from 0.2% to more than 100% among all 31 cases with previously documented ITD mutations, a 0.01% allele burden among 1/75 cases with previously reported ITD-negative results, and a 0.1% allele burden in 1 of 3 cases with previously reported ITD-negative results in the initial diagnostic specimens and ITD-positive results at the same size in follow-up specimens. These data indicate

that minor leukemic clones (allele burden of less than 1%) may become prominent clones later in the course of the disease. **Conclusions:** In summary, delta-PCR detects ITD mutations with improved sensitivity and may be useful for detection of minimal residual leukemia. Detection of minor clones with ITD mutation at the time of diagnosis may be clinically significant since they may become predominant in relapsed or refractory leukemias.

H23. Evaluation of Acute Myeloid Leukemia Specimens by Array CGH Reveals New Abnormalities across Prognostic Groups

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Introduction: The clinical heterogeneity of acute myeloid leukemia (AML) is well appreciated and cytogenetics play a key a role in predicting survival, with risk defined as good [e.g., t(8;21), inv(16), t(15;17)], intermediate [e.g., normal karyotypes, +8, t(3;5), t(9;11)] and poor [e.g., complex, 5q-, 7q-, other 11q23, inv(3)]. Although patients can be assigned to prognostic risk groups based on the cytogenetic findings, further refinement of these groups based on additional genetic findings should prove helpful for tailoring prognostic and treatment decisions. Microarray analysis (aCGH) of other hematological disorders has proven a powerful means to characterize disease, and the technology is gaining popularity both as a discovery tool and a diagnostic test, offering a higher resolution analysis compared to traditional cytogenetic approaches. We have utilized aCGH to analyze two series of cases with AML, reflecting a diversity of prior cytogenetic findings. **Methods:** We have developed the Signature OncoChip™ oligo-based microarray platform with genome-wide backbone coverage (1 probe/35 Kb) and higher density coverage for >1800 cancer features (1 probe/0.2-7.0 Kb) to detect copy number alterations (CNAs) for a diversity of hematologic disorders. aCGH was performed on two sets of AML specimens including a series of 63 AML cases (#1), all with known abnormal karyotypes representing those categories mentioned above, and a second series of 10 cases (#2) principally with normal karyotypes or novel balanced translocations, and generally poor outcome. **Results:** Analysis of a series #1 detected all prior cytogenetically recognized copy gains and losses in 57/63 (>90%) cases analyzed. Low level mosaicism (LLM) (5% to 20%) accounted for all abnormalities not detected. Results defined karyotypically complex cases and also identified non-recurrent CNAs across prognostic groups [e.g., deletions of 1q21.1 (*GPR89B*), 5q25.1 (*RANBP17*), 12p13.2 (*ETV6*), 13q14.2 (*RB1*), 16q21 (*CDH8*)]. For series #2, of 5 karyotypically normal cases, aCGH revealed multiple abnormalities in two of these cases (40%) [+8 with deletions 3p14.1 (*FOXP1, MITF*), 9q21.2->q33.2, 17q11 (homozygous *NF1*); and -7, +8, +11, +15]. For cases with novel balanced translocations, aCGH revealed additional novel alterations (80%) [e.g., deletions of 11p13 (*WT1, LMO2* and *CAPRIN1*), 17q21 (*WNT3*), and 15q14->q21.1 (*B2M*, *TP53BP1*, and *RASGRP1*) and partial tandem duplication of *MLL*]. **Conclusions:** aCGH analysis of AML cases identified all abnormalities previously detected by cytogenetics (excluding LLM). Additional alterations were identified across prognostic groups consistent with the higher resolution of the technology. Interestingly, cases with normal karyotypes or novel balanced translocations and generally poor outcome revealed the highest number of new findings.

H24. Detection of *RAS* Mutations in Multiple Myeloma Using Sequenom MassArray Platform

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Introduction: Multiple myeloma (MM) is an incurable hematologic malignancy with a three to four year median survival. Activating mutations in the *RAS* oncogene (*NRAS*, *KRAS*) are present in 30 to 40% of cases and are thought to represent an early event in the disease progression. Whole genome sequencing and other studies have shown that *RAS* family genes are the most frequently mutated genes in this disease. We investigated the use of mass spectrometry for the detection of specific *RAS* mutations in MM. **Methods:** Genomic DNA was extracted from BM aspirates from 19 patients with confirmed MM. We used Sequenom Mass Spectrometry Genotyping for specific mutations in codon 12, 13, 61 and 146 of *KRAS* and *NRAS*. The performance of this platform has been validated in our laboratory and the results are deemed accurate. The specific mutations are detected by amplification of the corresponding exons by PCR, followed by single base extension at the site of the point mutation. The extension product is detected by Tandem-Mass spectrometry on the Sequenom MassArray Spectrometer. **Results:** Seven cases (37%) were positive for *RAS* oncogene mutations. Four cases showed *KRAS* mutations: one codon 12 (G12D), two codon 61 (Q61L, Q61H) and one codon 146 (A146V) mutation. Three cases harbored *NRAS* mutations: one codon 13 (G13R) and two codon 61 (Q61R) mutations. **Conclusions:** Using Sequenom MassArray technology, we found *RAS* oncogene mutations in 37% of MM samples, similar to the frequency reported in the literature. This platform affords precise, rapid and reliable detection of specific *RAS* mutations and further studies investigating the significance of *RAS* mutations in combination with other genetic lesions in MM are planned. In addition to standard genomic analysis, individual sequencing studies have the potential to create a personalized approach to the diagnosis and treatment of MM.

H25. *NPM1* Mutation Occurs in a Subset of Ph+ AML Suggesting that this Entity Is Distinct from CML in Myeloid Blast Phase

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Introduction: The existence of *de novo* acute myeloid leukemia (AML) associated with t(9;22)(q34;q11.2)/BCR-ABL1, also known as Philadelphia chromosome-positive (Ph+) AML, is controversial. Investigators who doubt the existence of Ph+ AML suggest that criteria for distinguishing Ph+ AML from chronic myelogenous leukemia (CML) initially manifesting in a myeloid blast phase (BP) are not convincing. Other means of distinguishing Ph+ AML from CML-BP would be helpful. **Methods:** We searched the files of our hospital for AML cases associated with t(9;22)(q34;q11.2)/*BCR-ABL1* between January 1998 and January 2009. Patients with a history of chronic or accelerated CML, a CML-like picture after therapy, splenomegaly, or basophilia were excluded. We also identified a group of 12 patients with CML in myeloid BP with a documented antecedent CP during the same study period for comparison. *BCR-ABL1* fusion transcripts were assessed by real-time qRT-PCR. Mutational analyses of the *NPM1* (n=7) and *FLT3* (n=4) genes were performed using PCR/capillary electrophoresis. **Results:** We identified 7 patients with *de novo* Ph+ AML. There were 4 men and 3 women, 22-76 years of age (median, 66 years). Conventional cytogenetics showed t(9;22)(q34;q11.2) in all patients; qRT-PCR proved *BCR-ABL1* in all 6 cases assessed. Two of 7 (29%) patients had *NPM1* mutation; both were alive in molecular remission 20 and 57 months after diagnosis, respectively. *FLT3* was wild type in 4 cases tested, including both patients with *NPM1* mutation. None of 12 patients who had CML-BP had *NPM1* mutation. **Conclusions:** The detection of *NPM1* mutations in Ph+ AML supports the existence of Ph+ AML as an entity distinct from CML in myeloid BP. Although the study group is small, the frequency of *NPM1* mutation in Ph+ AML is similar to AML cases in general. It is also of interest that both patients with *NPM1* mutation responded well to therapy with molecular remission.

H26. A Photodiode Array for *KIR* Genotyping

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Introduction: In this study, we designed a technique for killer cell immunoglobulin-like receptor (*KIR*) genotyping by photodiode array (PDA). Genes encoding *KIR*s are variable among individuals. The detection methods for *KIR* genotyping have been developed using various technologies. **Methods:** The PCR-amplified biotinylated exon4, 5, and 9 were hybridized to the immobilized probe DNA on the photodiode surface, and the chip was incubated in an anti-biotin antibody-conjugate gold nanoparticle solution. The silver enhancement by the gold nanoparticles bound to the biotin of the target *KIR* genes precipitates silver metal particles at the chip surfaces, which block light irradiated from above. The resulting drop in output voltage depends on the amount of target DNA present in the sample solution, which allows the specific detection and the quantitative analysis of the complementary target DNA. **Results:** The PDA chip showed high relative signal ratios of *KIR* probe DNA hybridized to complementary target DNA, indicating an excellent capability in discriminating the presence and absence of the 16 *KIR* genes. **Conclusions:** This chip assay allows for efficient genotyping of the *KIR* locus.

H27. Clinical Evaluation of a Quantitative RQ-PCR Assay for *NPM1* Mutant Expression in Acute Myeloid Leukemia (AML) Using the Ipsogen *NPM1* MutaQuant RUO Kit

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Introduction: Mutant Nucleophosmin (*NPM1* mutant) mRNA may be a significant marker for classification, prognosis, and treatment decisions for AML patients. In addition, quantitative monitoring of *NPM1* mutant messenger RNA activity may assist providers to assess minimal residual disease (MRD) and molecular disease relapse well in advance of hematologic disease relapse, possibly extending the survival of these patients. Here we evaluated the performance characteristics of a commercially available assay, Ipsogen *NPM1*-A MutaQuant RQ-PCR for the quantitation of mutant *NPM1* in AML patients. **Methods:** Precision and linearity studies were carried out using the *NPM1* mutation positive OCI-AML3 cell line (German Collection of Microorganisms and Cell Cultures DSMZ, Germany) diluted in the CCRF (American Type Culture Collection, VA) cell line. Linearity was assessed by evaluating five dilutions (OCI-AML3 cells diluted in CCRF cells) in duplicate in 3 separate experiments on 3 separate days and an additional two duplicate dilutions on 2 additional days. Precision was assessed by evaluating a high and a low dilution tested in duplicate on 5 separate days. Clinical correlation studies were carried out using 30 peripheral blood and bone marrow samples that had been qualitatively tested for *NPM1* mutations at outside laboratories. Three normal peripheral blood samples were also evaluated. All results were normalized using *ABL* gene expression (i.e. *NPM1* copy number divided by *ABL* copy number multiplied by 100 and reported as %NCN; i.e. NCN = normalized copy number). **Results:** The relationship between the log%Normalized Copy Number vs. Ct was linear with an R2 value > 0.99 and efficiency greater than 90% (slope = -3.50) with a tested lower limit of quantitation of 10 OCI-AML3 cells/999,990 CCRF cells. For precision, the high and low dilutions were repeated in duplicate on 5 separate days yielding the following: 10 OCI-AML3 cells/999,990 CCRF cells — mean = 0.091%NCN, SD = 0.039, CV = 43.65 and 100,000 OCI-AML3 cells/900,000 CCRF cells — mean = 197.936%NCN, SD = 52.222, CV = 26.38. A 100 percent correlation between positivity and negativity was obtained for all clinical samples tested at outside laboratories. The 3 normal samples also tested as negative. **Conclusions:** The Ipsogen *NPM1* MutaQuant RUO kit is a good method for quantitation of mutated *NPM1* mRNA expression in patients with known *NPM1* mutation positive AML.

H28. Performance Variability of *BCR-ABL* Monitoring Tests: Impact of PCR Platform Standardization on Health Care Systems

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Introduction: This study examines inter- and intra-lab variability (and clinical consequence) among *BCR-ABL* monitoring tests. Three labs with the GeneXpert® Dx System (GX) using the Cepheid *BCR-ABL* Monitor Assay with an international scale (IS) conversion factor and 3 labs with lab-developed tests (LDTs) were compared in a system modeling CML patients (pts). **Methods:** K562 cell-spiked blood corresponding to BCR-ABL(IS) ratios ranging from ~10% to ~0.01% was tested. Scenarios included: 1) health care system (HCS) relying on 3 GX labs; 2) HCS relying on 3 LDT labs (without IS standardization). **Results:** Test of means showed no statistical difference between GX sites, whereas all LDTs differed significantly. The ratio of median measurements for each spike level (highest divided by lowest site) was less than 2 for the GX sites for all levels (<1.6 for spike levels >0.1% BCR-ABL(IS)) whereas LDT ratios ranged from 22 at a spike level of 0.13%, to ~10 for all other levels. The variability in the LDT-based HCS was considerably higher than that in the GX-based HCS. The LDT labs consistently reported either higher or lower values than the GX sites, indicating that inter-lab variability among LDTs could improve with IS standardization. Objective criteria for attainment of major molecular response (MMR) were applied against 100 virtual patient transcript profiles (VTPs) modeled after actual *BCR-ABL* pts observed in the IRIS clinical trial using the underlying data. Inter-lab pairwise discordance in MMR scoring ranged between 4% to 7% of VTPs within the GX HCS, 20% to 57% of VTPs within the LDT HCS, and 8% to 49% of VTPs between labs in the 2 different HCSs. Within the GX HCS, scoring per NCCN criterion (3-log reduction) vs IS criterion (≤0.1%(IS)) resulted in discordant interpretation of MMR among 2% to 13% of VTPs (intra-lab variability and MMR determination details for all sites will be presented). Using budget impact modeling, the economic benefits of a standardized platform were examined. For 100 pts monitored by guidelines, an estimated cost savings of $43,000 could be achieved over 1 year. **Conclusions:** Because inter-lab variation and differences in defining MMR could result in different interpretations for management of CML pts, these data underscore the need for PCR standardization. Automated platform standardization and adoption of the IS can improve *BCR-ABL* inter-lab variation. Because the standardized manufacturing and QC schema reduce the scope for test validation, test duplication, or QC panels, the standardized platform represents a potential cost-saving alternative.

H29. Cell Compartment Discrepancies in Hematopoietic Stem Cell Transplantation Chimerism Analysis

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Introduction: After treatment with an allogeneic hematopoietic stem cell transplant (HSCT), chimerism analysis is critical in ascertaining graft success, disease recurrence, and treatment complications such as graft versus host disease (GVHD). The utility of chimerism analysis in subsets of blood cells is not well studied. **Methods:** We studied chimerism analysis results retrospectively in 76 patients who had undergone HSCT for malignancy. Chimerism was determined by PCR amplification of short tandem repeats in bone marrow (BM) and unfractionated (UF) and/or CD3+ sorted cells from peripheral blood (PB). A significant discrepancy between UF and CD3+ compartments was defined as >10% difference in chimerism. **Results:** Fifty-two patients achieved complete donor engraftment by day 30 post HSCT. Twenty-four patients showed mixed chimerism and, among these, 14 showed significant discrepancies. In 10 patients (6 lymphoma, 4 leukemia), CD3+ cells of patient origin were present at a higher percentage than in UF cells, while 4 patients presented with a higher percentage of UF cells of patient origin. The latter 4 patients (all leukemia) showed poor clinical response (3 relapsed, 1 had graft failure). Consecutive analysis at 1-2 month intervals showed that patients with >50% CD3+ patient cells (3 patients total) in their PB by 6 months after transplantation had either graft rejection (1) or disease relapse (2). Those who presented with <50% CD3+ patient cells (6) eventually converted to complete donor chimerism. Discrepancies were transient (defined as <3 months) in 4 patients while 4 others demonstrated persistence of the discrepancy for >6 months. For those patients with paired BM, the patient chimerism in the BM more closely matched the UF than the CD3+ fraction. No correlation was found between rate of GVHD and/or graft failure and presence or level of discrepancy of mixed chimerism. **Conclusions:** We conclude that cell compartment discrepancy is a fairly common phenomenon in allogeneic HSCT patients. In about half of patients it is transient while others have longer lived cell compartment discrepancy. Analysis of bone marrow chimerism does not substitute for analysis of either cell compartment, as it fails to reliably predict their chimerism. Regardless of discrepancy, high levels of CD3+ patient cells in the peripheral blood appear to correlate with poor clinical outcome. In cases with discrepancy, a low level of CD3+ patient cells but higher UF patient cells correlates with relapse, although this group also contains a higher proportion of leukemia patients than the other group.

H30. High Resolution Melting Analysis as an Upfront Cost Effective Screening Method for Detection of *KIT* Exon 17 Mutations in Acute Myeloid Leukemia in the Clinical Laboratory

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Introduction: Gain of function mutations in Exon 17 of *KIT* occur at a low frequency in acute myeloid leukemia (AML) and are associated with poor prognosis. A review of clinical test database at our diagnostics laboratory showed that mutations in exon 17 of *KIT* were detected in only 70 of 2975 (2.4%) specimens by Sanger sequencing. To reduce the effort and costs associated with the sequencing of such low frequency mutations, we developed and validated a high resolution melting analysis (HRMA) screening method to detect variants in exon 17 of *KIT*. **Methods:** A total of 127 peripheral blood or bone marrow aspirates from patients with AML previously tested by Sanger sequencing for *KIT* exon17 mutations were included in this validation study. The study cases were selectively enriched for *KIT* mutations (N=52; codon 813(N=4), codon 816 (N=36), codon 822 (N=4) and codon 798(N=8)). HRMA was performed using 50 ng of DNA on the LightCycler® 480 (Roche, Indianapolis, IN). HRMA-PCR products were sequenced in all cases. The assay sensitivity was determined by using serial dilution of positive cell line (Kasumi) DNA into a negative cell line (HL60) DNA. **Results:** Variant calls were made by HRMA in 62/127 cases that included all 52 cases previously shown to have mutations by Sanger sequencing. Sanger sequencing of the post HRMA product also confirmed mutations in these 52 samples. The remaining 10 HRMA variants were wild type by Sanger sequencing (false positive rate: 7.9%). Notably, there were no false negative calls by HRMA. HRMA accurately genotyped 92.1% of the

cases. The sensitivity of the HRMA assay was 10% in our serial dilution studies. Post-validation application of HRMA in routine diagnostics workflow detected variants in 32/303 (10.6%) of AML samples, resulting in 89.4% reduction in Sanger sequencing volume. **Conclusions:** HRMA is a reliable screening tool that provides an efficient alternative to Sanger sequencing for low frequency mutations resulting in time, cost and labor savings and improving turnaround time for majority of cases.

H31. *GUSB* or *BCR*, but not *ABL*, Is the Best Internal Control Gene for *BCR-ABL1* Quantification

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Introduction: The control gene for *BCR-ABL1* quantification serves as the internal caliber to control for RNA loss from the step of cell preparation to PCR. It also controls for pre-analytical RNA degradation. As the control for both RNA quantity and quality, the control gene is the single most important determinant for accurate *BCR-ABL1* quantitative assessment. Several years ago, the International *BCR-ABL* RQ-PCR Standardization Group recommended that *ABL*, *BCR* or *GUSB* be used as the control gene for *BCR-ABL1* quantification (Blood, 108, 28-37, 2006). However, our analysis of the recent CAP surveys (presented before the International *BCR-ABL* RQ-PCR Standardization Group in December 2009) showed, with data collected from more than 100 participating labs, that *ABL*-controlled *BCR-ABL1* levels provide significantly different quantitative values from those that are expected for the survey samples. In comparison, *BCR*- or *GUSB*-controlled *BCR-ABL1* levels were accurate. This observation remains true for several recent CAP surveys. We have been interested in the identification of the best control gene. Previously, we showed that *GUSB* and G6PD are the best control genes by comparing 14 candidates (including *ABL* and *GUSB*, but not *BCR*) using the six most relevant criteria (J Mol Diagn 2006, 8:231-239, 2006 and J Mol Diagn 2006, 8:385-389). Owing to the more frequent occurrence of G6PD mutations in the hereditary disorder of G6PD deficiency, *GUSB* was recommended as the most suit*ab*le control gene. **Methods:** In the current study, using serial dilutions of cell lines (K562 into HL60) and primary cells (CML into normal total white cells), we determine the dynamic range of *BCR-ABL*1/*ABL*1, *BCR-ABL*1/*GUSB* and *BCR-ABL*1/*BCR*. **Results:** We show that *ABL*-controlled *BCR-ABL1* quantification exhibits a much narrower linear range than *BCR*- or *GUSB*-controlled *BCR-ABL1* quantification. **Conclusions:** By comparing and contrasting the three recommended control genes, our results further demonstrate that *GUSB* or *BCR*, but not *ABL*, is the best internal control gene for *BCR-ABL1* quantification. The limitations of using *ABL* as the control gene, in compliance with the recommendations by the International *BCR-ABL* RQ-PCR Standardization Group, will be shown and discussed.

H32. High Resolution Whole Genome Scanning for Acquired Copy Number and Copy Neutral Alterations in Cytogenetically Normal Multiple Myeloma

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Introduction: Multiple myeloma (MM) is the second most common hematological malignancy, characterized by uncontrolled accumulation of clonal plasma cells. Clinical and cytogenetic features have been the major prognostic markers for the overall management of this incurable disease. Cytogenetically, the prognostication has been significantly hampered to an extent by the small number of monoclonal plasma cells and the low proliferative activity in the bone marrow. Although FISH analysis has been a valuable tool in clinical diagnostics, the fast emerging genome-wide chromosomal microarray analysis (CMA) has provided an improved understanding of MM pathogenesis. **Methods:** Our goal was to identify the global genomic aberrations using high resolution SNP array in 20 cytogenetically normal MM patients. To aid identification of the true somatic mutations in tumor genome we used patient's matched normal genome derived from skin biopsies. This approach is very important to determine which copy number alterations are inherited versus acquired. DNA was isolated both from CD138 positively selected plasma cells and skin. Affymetrix SNP 6.0 microarray was used to deduce copy number and copy neutral alterations. The data was analyzed using Nexus copy number analysis software. All the samples passed the SNP QC and the call rate were >94%. **Results:** The genomic aberrations in the abnormal patients well correlated with the reported cytogenetic analysis. Interestingly, all the cytogenetically normal patients showed abnormality throughout the genome in our cohort. Genomic abnormalities were present in all the chromosomes with over 25% gains in 1q, 3, 5, 7, 9, 11, 15, 18, 19, 21 and deletions in 1p, 6q, 8, 12p, 13, 14, 16q and 22. In particular, deletion of chromosome 1p, 6q, and 8p and amplification of chromosome 11 were significantly different between these groups. We also observed LOH on chromosome 1, 9, 11, 16 and 19 in the cytogenetically normal group. Overall our results emphasize the growing importance of the CMA in identifying the cytogenetically unidentified genomic aberrations. In conclusion, we identified novel genomic abnormalities in nearly all the cytogenetically normal patients and our data correlates well with the known pattern of genomic aberrations in MM pathogenesis. **Conclusions:** The contextual relevance of these mutations will be better understood with analyses of additional clinically and demographically stratified cytogenetically normal MM patients. Whole genome scanning for copy number alterations will help in precisely prognosticating MM, in risk based stratification of treatment and perhaps survival.

H33. Re-evaluation of Molecular Diagnostic Tests for T-Cell Receptor Clonality in Taiwanese Populations

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Introduction: Monoclonal expansion of T-cell receptor (TCR) genes is an important marker for T-cell malignancy. The genetic features shown by TCR gene rearrangements offer useful markers to distinguish lymphoid hyperplasia from malignancy from TCL, especially for cases with atypical histopathological features. Therefore, the molecular diagnosis of TCR gene clonality has been used in TCL diagnosis for years. We used BIOMED-2 protocols to detect the clonality of these genes in TCL in southern Taiwan. Heteroduplex analysis was used after PCR reactions. TCR clonality rate was 76% (19 in 25 cases) for TCL, which was lower than previously reported in western populations (95%). We hypothesized that the TCR genes present sequence polymorphisms between Taiwanese and western populations and some BIOMED-2 primers might not be suitable for Taiwanese cases. **Methods:** We performed 5'-RACE cDNA amplification approach, followed with ligation-mediated PCR, and amplified cDNAs of the various TCRβ genes in healthy Taiwanese individuals. The cDNA products of these TCRβ genes were sequenced. **Results:** We found that some variable (V) and joining (J) genes presented sequence variations between our subjects and western populations, based on the sequence comparison of the TCRβ genes in our subjects and the ones in the databank of the International ImMunoGeneTics Information System® (IMGT, Geneva, Switzerland). We are now initiating a next-generation sequencing approach to gain all of the TCR gene sequences in Taiwanese populations. **Conclusions:** The sequencing results will be used to develop a new and improved set of specific PCR primers for TCR clonality in Taiwan, probably also in other Asian countries.

H34. SNP Copy Number Array Is a Practical Alternative to Cytogenetics in the Detection of Common and Cryptic Mutations in Patients with Leukemia and Lymphoma

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Introduction: While morphological analysis of neoplastic cells provides significant insight into disease, understanding the molecular changes associated with neoplastic transformation provides critical prognostic information and guides appropriate treatment. Cytogenetic approaches such as G-band and FISH analysis detect genetic abnormalities that commonly occur in hematolymphoid malignancy, but they have limited resolution. In order to provide comprehensive molecular characterization of copy number abnormalities in hematological malignancies, we validated the use of Illumina HumanOmni1Quad SNP copy number arrays (SNParray) to determine cryptic and gross genetic alterations, including copy neutral loss of heterozygosity, in routine clinical specimens. **Methods:** 45 blood and bone marrow specimens received by the Oncology Cytogenetics Laboratory at Emory University for routine FISH and G-band analyses were identified. Excess samples were stored at 4°C and submitted to the CLIA-certified Cancer Genomics Shared Resource at Emory's Winship Cancer Institute. DNA was extracted and quality was assessed using a NanoDrop spectrophotometer. Whole genome copy number analysis was performed using the SNParray that contains more than 1.1 million probes and has a mean probe spacing of 2.4 kb. Data analysis was performed using the Illumina KaryoStudio software and aligned to NCBI human build 36.3 (hg18). Microarray results were compared to G-band and FISH results. **Results:** SNP copy number analysis of the 45 samples was concordant with deletions and duplications identified by FISH and/or G-banding. This included a broad range of abnormalities seen in ALL, AML, multiple myeloma and follicular lymphoma. In addition, the SNP array identified a cryptic *IKZF1* mutation not previously detected in a patient with relapsed ALL. Further analysis demonstrated several other samples with clinically relevant copy number abnormalities undetectable by routine G-band or FISH analysis. **Conclusions:** With the assistance of a CLIA certified genomics core, we have implemented a clinical diagnostic workflow for molecular pathology and oncology cytogenetics services at Emory University. While the clinical utility of SNP copy number arrays for cancer diagnostics continues to be explored, we are now able to provide a broad-range clinical and translational resources to complement routine cytogenetic and

molecular genetic analysis. Although clinical reporting of SNP copy number data is currently restricted to known and/or actionable abnormalities, collection of these clinical genomic datasets will provide an invaluable resource for future diagnostic, prognostic and therapeutic correlates.

H35. Detection of *MPL* Mutations by a Novel Allele-Specific PCR-Based Strategy

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Introduction: *MPL* gene mutations occur in primary myelofibrosis (PMF) and essential thrombocythemia (ET) at a frequency of approximately 10% and 3%, respectively. Testing for *MPL* mutations can aid in the diagnosis of these myeloproliferative neoplasms and is recommended in patients with suspected PMF or ET who lack the *JAK2* V617F mutation. In this setting, the presence of an *MPL* mutation establishes a clonal myeloproliferation and meets a major diagnostic criterion in the most recent revision of the WHO classification of ET and PMF. Several methods have been developed to detect *MPL* mutations, with both Sanger sequencing and high resolution melting (HRM) being commonly utilized. Our aim was to develop and implement a robust and sensitive *MPL* mutation test that could be easily deployed in our laboratory. **Methods:** A novel allele-specific PCR assay capable of detecting four *MPL* mutations (W515L, W515K, W515A, S505N) was developed and optimized. The assay utilizes a two-tube multiplexed PCR format with detection by capillary electrophoresis. Genomic DNA from 15 reference specimens (5 positive, 10 negative) was tested and results compared to those obtained by Sanger sequencing. Results from 111 consecutive specimens referred to our laboratory for testing were also reviewed. **Results:** The multiplexed allele-specific assay reliably detected each of the *MPL* mutations down to 2.5% mutant allele. The 5 mutation positive reference specimens were readily detected by both the allele-specific assay and Sanger sequencing. Mutation levels in the reference specimens ranged from ~50% to 95%. Of the 111 specimens received for *MPL* testing, 104 tested negative and 7 positive (all W515L). Two of the W515L mutations were present at less than 10% mutant allele and were not easily detected by Sanger sequencing. Results from all allele-specific PCR tests were easily interpreted. **Conclusions:** Allele-specific PCR represents a viable platform for *MPL* mutation testing. Our assay provides a simple, efficient, and sensitive method that is capable of detecting the vast majority (>95%) of *MPL* mutations associated with ET and PMF. Sanger sequencing may have insufficient sensitivity to detect cases with low-level *MPL* mutations.

H36. Acute Myeloid Leukemias with Isolated Del(6p): A Rare Entity with a Common Deleted Region Confined to 6p23~P24 and Infrequent Mutations of *FLT3*, *RAS*, *KIT* and *NPM1*

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Introduction: Recurrent chromosomal abnormalities in acute myeloid leukemia provide valuable pathogenetic and prognostic information. Isolated abnormalities of chromosome 6p in acute myeloid leukemia are rare. Until now, only 3 cases of *de novo* AML with isolated del(6p) have been reported. The frequency of genetic mutations in *NPM1*, *FLT3*, *RAS* and *KIT* in this group is not known. We report the clinicopathologic and molecular genetic features of 6 additional cases of AML with del(6p) as the sole abnormality. **Methods:** All cases of acute leukemia with isolated del(6p) between 1997 and 2011 in the cytogenetic database at our institution were retrieved. Bone marrow aspiration and biopsy specimen slides were reviewed. Clinical information was obtained from the medical records. Genomic DNA from bone marrow aspirate samples was amplified by PCR followed by mutational screening for *NPM1* (Capillary electrophoresis), *FLT3*, *KIT* (direct Sanger sequencing), and *RAS* (pyrosequencing). **Results:** Six cases of acute leukemia with isolated del(6p) were identified, all of which showed myeloid immunophenotype. These included 5 women and 1 man with a median age of 58 years (range, 39~68 years). The initial blood counts demonstrated a median white blood cell count was 4.35 K/UL (range, 1.2~6.8), median hemoglobin was 9.9 G/DL (range, 9.8~11.4), and median MCV was 82 FL (range, 79~100). The median platelet count was 111 K/UL (range, 26~293). The median bone marrow blast count was 43% (range, 25~60%). Morphologically, dysplasia was most commonly seen in the granulocytic lineage in the form of Pseudo-pelger Huet neutrophils. The cases were classified as AML with myelodysplasia related changes (n=3), AML with maturation (n=2) and therapy related AML (n=1) using the WHO classification. The common deleted region all cases was small and confined to 6p23~p24. Mutation studies on 4 cases with available DNA revealed no mutations of *FLT3* (n=3), *RAS* (n=4) or *KIT* (n=3). *NPM1* mutation was seen in 1 out of 4 cases tested. All but 1 patient died with a median survival of 10 months (7~18 months). The patient with *NPM1* mutation is alive at 34 months follow-up. **Conclusions:** AML with isolated del(6p) is characterized by a distinct female predominance in older adults and frequent granulocytic dysplasia. The common deleted region (6p23~p24) in all cases may harbor a potential tumor suppressor gene. None of the cases demonstrated any mutations in *FLT3*, *RAS* or *KIT*. Mutation in *NPM1* gene was present in the patient who was alive at last follow-up.

H37. Pros and Cons of Cancer Microarray Testing: Experience from Validation on Hematological Malignancies

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Introduction: Microarray testing has become the standard of care in the diagnosis of constitutional chromosome abnormalities in patients with congenital abnormalities and developmental disorders. Various microarray platforms have contributed greatly to the understanding of global somatic genetic imbalance associated with cancer by many research studies. Application of clinical testing with the microarray technology has gained popularity. The establishment of optimal practice standards and guidelines requires the experience from multiple testing centers. **Methods:** We have validated 71 specimens from 66 patients with hematological malignancies, including 43 myeloid disorders and 18 lymphoid disorders, using four different commercial array platforms. Among these, 15 samples were tested on at least two different types of arrays. Data analysis was performed both with the manufacturer's software and with the Nexus software examining copy number aberrations (CNA) as well as loss of heterozygosity (LOH). **Results:** Compared with known cytogenetic abnormalities, microarray testing was able to detect additional CNA in 57% of all specimens and copy neutral LOH in 21% of specimens tested with SNP-arrays, highlighting the advantage of significantly improved resolution of this technology in general and the unique strength of the SNP-arrays. As expected, balanced translocations, low-level mosaicism, and clonal evolution could not be detected (14%). One sample with a balanced translocation, however, demonstrated deletions of breakpoint regions on both chromosomes involved. All platforms were similar in performance although one missed a small deletion. In acute lymphocytic lymphoma, the most frequent aberration was 9p deletion encompassing the p16 gene locus (38%), followed by deletions of 1p, 6q, 9q and 17p. All but 17p deletion appeared to be associated with patients who have deceased in our cohort. For myelodysplasia and acute myeloid leukemia, the most frequent CNAs were the typical 5q-, 7q-, 13q-, 20q- and trisomy 8 (30% to 40%). Deletion 7q was clearly more prevalent among deceased patients while 20q- was only detected in patients who survived longer. **Conclusions:** Despite the recognized limitations, microarray testing for cancer improves detection sensitivity in general and allows identification and better characterization of novel prognostic markers.

H38. A Novel Nanofluidics-Based Approach for Simultaneous and Quantitative Detection of Multiple Recurrent Translocations in Hematologic Malignancies

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Introduction: Chromosomal translocations that result in chimeric transcripts are useful as molecular markers for a variety of hematologic malignancies. Current molecular approaches to interrogate these markers include quantitative (qPCR) assessment of each translocation individually or qualitative assessment of a predefined set of translocations as in Signature® LTx (LTx) Panel (Asuragen, Austin, TX). These approaches require 1-2 μg of RNA/sample. Alternatively, FISH can be used, but this approach also is limited by the evaluation of one translocation at a time and is both labor-intensive and costly. To overcome these limitations, we developed a novel nanofluidics-based approach for simultaneous and quantitative detection of multiple translocations using nanograms of RNA in nanoliter reaction volumes. **Methods:** We utilized Integrated Fluidic Circuit (IFC) arrays (Fluidigm®, San Francisco, CA) and Taqman probe-based qPCR to simultaneously detect 10 leukemia-associated fusion transcripts: b2a2/b3a2 and e1a2 forms of *BCR/ABL1* [t(9;22)(q34;q11.2], *RUNX1/RUNX1T1* [t(8;21)(q22;q22)], *E2A/PBX1* [t(1;19)(q23;p13.3)], *ETV6/RUNX1* [t(12;21)(p13;q22)], *PML/RARα* long and short forms [t(15;17)(q22;q12)], *CBFβ* A and D variants [inv(16)(p13.1q22)], and *MLL/AF4* [t(4;11)]. ABL was assessed as an internal control. A total of 112 peripheral blood or bone marrow aspirate specimens (AML 67, ALL 35, CML 7, other: 3), previously tested by qualitative LTx panel were analyzed by the nanofluidic 48X48 IFC array format that allows analysis of 48 samples and 48 targets/ sample. A total of 60 ng of RNA was used for each sample. Each of the targets was analyzed in triplicate. Plasmids containing known amounts of fusion sequences were utilized to generate standard curves for quantification. Results were also compared with available karyotype and FISH results. **Results:** IFC arrays correctly detected fusion transcripts with high specificity. Data output was easy to interpret and results were highly reproducible. FISH and IFC arrays were in concordance in all but

two cases, where IFC arrays, but not FISH, detected a translocation. Overall concordance between nanofluidics and LTx screen was 99.7% across all translocations. Unlike the qualitative screen, IFC arrays do not require a second qPCR assay for quantification. **Conclusions:** Simultaneous and quantitative detection of multiple recurrent translocations using nanofluidics arrays provide an efficient and cost-effective tool for rapid screening and classification of newly diagnosed leukemias and, for quantitative monitoring. Since each transcript is analyzed in individual wells, additional targets can be easily added to the panel without altering existing assays. Similarly, more than one internal control can be tested when necessary.

INFECTIOUS DISEASES

ID01. Evaluation of the BD MAX GBS Assay to Detect Group B Streptococcus in LIM Broth Enriched Antepartum Vaginal-Rectal Swab Specimens

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Introduction: Group B Streptococcus (GBS) remains the leading cause of neonatal morbidity and mortality in the United States. Traditionally, the Centers for Disease Control and Prevention (CDC) have recommended antepartum culture-based screening at 35 to 37 weeks gestation to detect GBS colonization. Revised CDC guidelines now support the use of molecular-based methods for antepartum screening. In August 2010, the BD MAX GBS assay (Becton Dickinson) was approved by the FDA for detection of GBS from LIM broth enriched antepartum vaginal-rectal swabs. This assay is performed on the BD MAX system - a fully automated, bench-top instrument integrating sample preparation, nucleic acid extraction, real-time PCR-based amplification, and detection of the *cfb* gene of GBS. The goal of this study was to determine the test performance characteristics, approximate cost per test, and work-flow efficiency of the BD MAX GBS assay and CDC-recommended culture methods. **Methods:** Vaginal-rectal swabs, collected from women (n=200) at 35 to 37 weeks gestation, were plated to modified Granada agar (MGA: Northeast Laboratories), then inoculated into LIM broth (Becton Dickinson). Following 24 hr incubation MGA was observed for GBS (orange colonies); if negative, LIM broth was plated to sheep blood agar (Becton Dickinson) for detection of GBS (hemolytic and non-hemolytic) over the next 48 hr and confirmed by conventional methods (catalase and CAMP). For the BD MAX GBS assay, 15 µL of LIM broth at 24 hr incubation was tested according to the manufacturer's instructions. **Results:** Of the 200 swab specimens tested, 42 and 154 were positive and negative, respectively, by both real-time PCR and culture. Four specimens yielded discrepant results and were tested by an alternate real-time PCR method (GeneOhm Strep B, Becton Dickinson). Following discrepant analysis, the BD MAX GBS and culture-based tests yielded identical sensitivity and specificity (97.7% and 100%). Throughput on the BD MAX GBS assay was about 3 hours for 24 specimens. The approximate cost and hands-on-time per test was: BD MAX GBS ($27, 1.5 min); culture ($4.29, 3.3 min). **Conclusions:** The BD MAX GBS assay performed equivalent to our culture-based method and concomitantly reduced the amount of required hands-on-time by 50%. The BD MAX GBS assay is an efficient, stream-lined alternative approach for antepartum GBS screening with significant advantages over traditional culture-based methods.

ID02. Evaluation of the Nanosphere Verigene® RV+ for the Detection of Influenza A with Subtyping, Influenza B, and Respiratory Syncytial Virus with Subtyping from Nasopharyngeal Swabs

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Introduction: Multiplexed real-time RT-PCR is a popular method for rapid detection of respiratory viruses from clinical specimens because of high diagnostic accuracy and improved turnaround-time. Nonetheless, real-time RT-PCR is labor intensive, requires batch testing, and requires specialized personnel and equipment. Verigene® Respiratory Virus Plus Nucleic Acid Test (RV+) is a recently FDA-Cleared RT-PCR assay with microarray based detection for a fully automated process from nucleic acid extraction to result generation in <2.5 hours. We aimed to compare the performance of the Verigene® RV+ with several real-time RT-PCR assays for detecting Influenza A with subtyping, Influenza B, and respiratory syncytial virus (RSV) with subtyping. **Methods:** Nasopharyngeal and throat swabs were collected from consecutive patients as part of routine clinical care from years 2008 to 2010. These samples were originally evaluated for Influenza A with subtyping, Influenza B, RSV with subtyping by Prodesse ProFlu+/ProFlu-ST (Waukesha, WI), Luminex xTAG RVP (Austin, TX), and CDC test kit (Atlanta, GA). The unused portion of these samples was archived. We utilized archived samples to evaluate Influenza A with subtyping, Influenza B, and RSV with subtyping using Verigene® RV+ (Nanosphere, Inc., Northbrook, IL). All assays were performed according to the manufacturer's recommendations. **Results:** We compared the assays on a combined total 95 respiratory specimens: 77 nasopharyngeal swabs, 9 throat swabs and 9 viral cultures. The sensitivities of the Verigene® RV+ were as follows: 97.4% (38/39) for Influenza A detection only with 91.6% (11/12) for H3 subtyping, 100% (11/11) for H1 subtyping, and 87.5% (14/16) for 2009H1N1 subtyping; 100% (11/11) for Influenza B detection; and 100% (20/20) for RSV detection only with 100% (5/5) for A subtyping and 100% (4/4)for B subtyping. The specificity of the Verigene RV+ was 100% (25/25 negative samples). **Conclusions:** Our results suggest that Verigene® RV+ performs well for detection of Influenza A with subtyping, Influenza B, and RSV with subtyping in a veteran population. From our experience, the Verigene® RV+ assay is desirable for a laboratory seeking a test of "moderate complexity" that can be performed by the general laboratory staff with minimal hands-on required.

ID03. Evaluation of Cervista™ HPV 16/18 Genotyping Assay: A Single Institute's Experience

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Introduction: Infection with high-risk (HR) subtypes of human papillomavirus (HPV) is associated with cervical cancer and precancerous cervical lesions. HPV types 16 and 18 are recognized as both highly oncogenic and persistent. The HPV 16/18 genotyping is recommended by the American Society for Colposcopy and Cervical Pathology (ASCCP). Our lab is the first in Houston, TX to use the FDA-approved Cervista™ HPV 16/18 assay for clinical diagnosis. We have analyzed the assay data. The correlation between the molecular and cytological results is also reviewed. **Methods:** The cervical specimens were obtained from the Outreach OB clinics in Houston area and were collected in PreservCyt® Solution. The Genfind™ DNA Extraction Kit was used for sample preparation. A Cervista HR HPV screening assay was initially performed to determine the presence of HR HPV. The Cervista™ HPV 16/18 genotyping was followed using the ASCCP guideline. The corresponding cytological findings were obtained from the medical records. **Results:** 255 HR HPV positive samples were tested for HPV 16/18 from September 2010 to April 2011. 63/255 were positive for HPV 16 (≈25%). 9/255 were positive for HPV 18 (3.5%). 1/255 was positive for both HPV 16 and 18 (≈0.4%). 182 other samples were tested negative for both HPV 16 and 18 (71%). Among the HPV 16 positives, there were 11 ASCUS, 2 ASC-H, 10 LSIL, 2 HSIL, and the rest 38 cases were normal on cytology. Among the HPV 18 positives, there were 2 ASCUS, 4 LSIL, 1 HSIL, and 3 of normal cytology. The specimen positive for both HPV16 and 18 was found to be LSIL. The prevalence of HPV16 among women with ASCUS in our patient population is ≈17.5% (11/63), which is comparable with the manufacture report. However, the prevalence of HPV 18 with ASCUS cytology is 20% (2/10) in our patients, which is significant higher than reported by the manufacture. 60% (38/63) and 33% (3/9) of our patients were positive for HPV 16 and HPV 18, but with normal cytology, respectively, indicating a high oncogenic potential associated with a HPV infection in our patient population. **Conclusions:** The Cervista™ HPV 16/18 genotyping assay has been reviewed after 8-month clinical application in our molecular laboratory. The molecular and cytological correlation has been analyzed. The prevalence of HPV 16 and 18 with ASCUS cytology is estimated for our patient population. The information is essential for the quality assessment.

ID04. The Internal Control in the Cervista HPV HR Assay Identifies Clinical Specimens with Insufficient Cellularity

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Introduction: Detection of high-risk HPV in cervical cytology specimens is widely accepted as an important part of cervical cancer screening. As a screening test, HPV detection assays must have a very high sensitivity, as false-negative results fail to identify women who may be at increased risk of developing cervical cancer. The internal control in the Cervista HPV HR assay is intended to distinguish truly negative results from false negative results caused by inadequate specimen cellularity. Here we review our data from specimens that were unsatisfactory for cytologic evaluation in order to determine whether the internal control truly identifies specimens with insufficient cellularity for testing. **Methods:** We reviewed our database for specimens that were unsatisfactory for cytologic evaluation and underwent HPV testing. We identified 181 specimens tested using the Cervista HPV HR assay (Hologic Inc.), and 172 specimens tested using the hybrid capture 2 assay (hc2, Qiagen). For each cohort, we reviewed the cause of the unsatisfactory cytology and the results of HPV testing. **Results:** In our Cervista cohort, 94 (51.9%) specimens were unsatisfactory for cytologic evaluation due to low cellularity. Of these, 51 (54.3%) gave an insufficient signal from the internal control, and 43 (45.7%) reported a result (40 negative, 3 positive). The remaining 87 specimens (48.1%) were unsatisfactory for cytologic evaluation for reasons other than cellularity (interfering substance, excessive lubricant, etc.). Of these, 14 (16.1%) gave an insufficient signal from the internal control, and 73 (83.9%) reported a result (66 negative, 7 positive). All specimens with an insufficient internal control signal would have been reported as negative in the absence of the internal control. In our hc2 cohort, 82 (47.7%) specimens were unsatisfactory for cytologic evaluation due

to low cellularity; 78 (95.1%) tested negative, 4 (4.9%) positive. Ninety specimens (52.3%) were unsatisfactory for cytologic evaluation for reasons other than cellularity; 77 (85.6%) tested negative, 13 (14.4%) positive. **Conclusions:** In specimens satisfactory for cytologic evaluation, our laboratory's rate of low internal control signal in the Cervista HPV HR assay is approximately 1.1%. In contrast, in specimens with inadequate cellularity for cytologic evaluation, our rate of low internal control in this study was 54.3%. The hc2 assay does not have an internal control, so all specimens tested with hc2 reported a result. Our data indicate that the internal control in the Cervista HPV HR assay does identify specimens with insufficient cellularity, and likely reduces the risk for false negative results.

ID05. SeptiFast for Diagnosis of Sepsis in Severely Ill Patients from a Brazilian Hospital

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Introduction: Sepsis is a leading cause of morbidity and mortality in hospitalized patients worldwide. Conventional blood culture (BC) is the gold standard for pathogens detection, but time required to results can range from 1 to 5 days depending on the organism. Recently, molecular methods for diagnosis of bloodstream infections were developed and are being used as an adjunct to traditional methods for faster and accurate results. In this study we tested and validated a multiplex real-time PCR for simultaneous detection of 25 common pathogens (bacteria and fungi). **Methods:** A prospective study was performed in 114 cases of critically ill patients from the Intensive Care Unit (ICU) of a private hospital. The study was conducted from 12/2008 to 10/2009 and was approved by the institution ethical committee. Blood samples were simultaneously collected for PCR and BC testing. PCR tests were performed in the LightCycler 2.0 System (LCS) and analyzed by the SeptiFast software (Roche Diagnostics). This assay amplifies the internal transcribed spacer (ITS) region between the 16S and 23S ribosomal DNA sequences of Gram-positive and Gram-negative bacteria, and the 18S and 5.8S ribosomal DNA sequence of fungi. Conventional BC was performed using BACTEC Plus Aerobic/F and BACTEC Plus Anaerobic/F bottles. All bottles were monitored by BACTEC 9240 blood culture system (BD). **Results:** Among the 114 cases, LCS was positive result in 23 (20.2%) and BC in 17 (14.9%). A total of 27 cases (23.7%) were positive by one of the two assays. In 12 patients, the same pathogen was identified. From the 15 discordant results, 4 patients were positive only with BC (2 *S.epidermidis* and 2 *P. cepacea*), 10 were positive only with LCS (5 *P. aeruginosa*, 1 *C. tropicalis*, 1 *S. aureus*, 1 *C. glabrata*, 1 *K. pneumoniae* and 1 *E. cloacoe*) and in one patient different pathogens were identified (*K. pneumoniae* by LCS and *B. cepacea* by BC). This last patient had a positive culture result for *K. pneumoniae* in tracheal aspirate. Time for BC results was 5 days for negative results and 3.5 days for positive results. SeptiFast results can be achieved in less than 8 hours. **Conclusions:** The present study suggests that SeptiFast is an important complement for sepsis diagnosis. Both tests should be performed in patients from whom this diagnosis is suspected. SeptiFast allows a faster diagnosis of bacterial and fungal infections and may reduce the ICU time as well as allow the rationalization of antibiotics use.

ID06. The Need for Human Papillomavirus (HPV) Testing for Primary Anal Screening

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Introduction: Infection with oncogenic types of HPV is a well-established risk factor for the development of genital carcinoma. The prevalence of anal HPV infection among men who have sex with men (MSM) is very high. However, currently there is no consensus on routine testing for anal cancer screening among MSM. This study was designed to evaluate HPV testing results on normal cytology samples to assess the value of HPV testing for primary screening. **Methods:** A total of 1241 ThinPrep anal swab samples from MSM were screened for cytology between January 2010 and April 2011. There were 77 (6.2%) normal cytology samples selected for this study. A residual sample with 4-8 ml aliquots was first treated with STAR (Stool Transporting and Recovery) buffer then HPV DNA was isolated by using High Pure PCR template preparation kits (Roche). HPV DNA was identified by a semi-nested (MY11/MY09/GP6+ primers) PCR procedure. HPV genotypes, including 14 high-risk (HR: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and 2 low-risk (LR: 6, 11), were detected by a one-step multiplex PCR with HPV genotype-specific primers. Beta-globin and Aminolevulinate delta-synthase 1 were amplified as internal controls for PCR. Approximately 200 ng DNA was used for each PCR reaction. The HPV genotypes were identified by the expected sizes of the amplicons on the 4% NuSieve® 3:1 plus agarose gel (Lonza) by electrophoresis. **Results:** HPV DNA was detected in 53 of 77 (68.8%) normal cytology samples. There were multiple types found individually, including 2 (13.3%), 3 (17.0%), 4 (15.1%) and 3.7% for 5 and 6 types, respectively. There were 9 specimens with unknown types (17.0%) and 16 samples with a single type (30.2%). The HPV genotypes identified with frequency were 16 (24.5%), 18 (13.7%), 31 (7.5%), 33 (11.3%), 35 (9.4%), 39 (11.3%), 45 (5.6%), 51 (9.4%), 52 (16.9%), 56 (11.3%), 58 (16.9%), 59 (13.2%), 66 (11.3%), and 68 (15.0%). There was no inhibitory effect observed on the PCR reaction. **Conclusions:** A very high percentage of HR HPV genotypes was detected in normal cytology samples from MSM. Our study demonstrated that a negative HPV test result has a better negative predictive value than a normal cytology result. We propose that HPV testing replace cytology testing for primary anal sample screening.

ID07. Analysis of External Run Control QC for Quantitative CMV and EBV Viral Load Assays with Automated Extraction and PCR Setup

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Introduction: The analysis of run control data for QC of quantitative molecular virology testing is performed to monitor assay accuracy and precision, however; current Laboratory Information Software (LIS) is limited in the ability to collect, analyze, and display run control QC data. Automation of manual sample extraction using the QIAcube and PCR setup using the CAS-1200 liquid handling system (QIAgility) was previously implemented to maintain accurate and reproducible setup for our in-house quantitative CMV/EBV real-time, TM ASR assays (Qiagen-Artus), relatively new tests for which there is little information regarding performance in a clinical setting. **Methods:** External run control data was collected over a two year period using CMV and EBV OptiQuant™ viral controls obtained from Acrometrix (Benicia, CA). The results for two levels of control ($5x10^3$ and $5x10^5$ for CMV, 10^4 and 10^6 copies/mL for EBV) were entered in Excel spreadsheets (Microsoft, Redmond, WA) and log10 transformed in a statistical approach to generate Levey-Jennings charts based on the mean +/- 3 SD. Data analysis was also performed using eQuality™ software from SeraCare (Milford, MA). **Results:** Statistical analysis demonstrated differences in the reproducibility of the external run controls between the CMV and EBV viral load tests, even though both assays are run together in the same 96 well plate of an AB7900 analyzer. The CMV assay $5x10^3$ control was more reproducible (5-6%CV) than the EBV assay 10^4 control (8-11%CV) using either an Auto-Ct, or a fixed, manual Ct method in an attempt to improve the EBV assay reproducibility. Consistent mean values obtained from the external run controls in both the CMV and EBV assays indicate that accuracy was maintained within 0.2 log10 on an annual basis. **Conclusions:** Differences in the reproducibility of external run controls at the low level of control ($5x10^3$ for CMV, 10^4 copies/mL for EBV) of two commercial quantitative real-time PCR assays for CMV and EBV viral load were noted, where the CMV assay was more reproducible than the EBV assay despite automated extraction and PCR setup utilized for both assays. The differences observed do not seem to be associated with the run control lot, nor with the assay set up.

ID08. Performance Validation of Roche Cobas AmpliPrep/Cobas Taqman HIV-1 Test Version 2.0 (CAP/CTM v2.0): Comparison to CAP/CTM v1.0 and Abbott m2000 Real-Time HIV-1 Assay

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Introduction: Since we implemented Roche CAP/CTM v1.0 assay for HIV-1 viral load quantification in late 2008, we have encountered reports from clinicians regarding increased numbers of low positives or "viral blips." With the FDA approval of Roche dual-target assay, CAP/CTM v2.0, we performed a comparison study of CAP/CTM v2.0 with CAP/CTM v1.0 and Abbott m2000 real-time HIV-1 assay, with a particular focus on the most clinically relevant low positive specimens. **Methods:** Linearity, accuracy, and precision of CAP/CTM v2.0 were verified by analyzing five levels of Acrometrix Valiquant controls in triplicate on 4 separate runs. Analytic sensitivity of CAP/CTM v2.0, CAP/CTM v1.0 and m2000 was evaluated with EDTA plasma spiked with 25 or 100 copies/mL control material. Clinical specificity was evaluated by testing 35 known HIV negative donors. Parallel testing of 76 clinical samples was performed on CAP/CTM v2.0 and CAP/CTM v1.0. Correlation was evaluated using Deming regression and calculated bias. Additionally, 25 patient samples with low positive HIV viral load (<200 copies/ml) by CAP/CTM v1.0 were cross-tested with CAP/CTM v2.0 and m2000. Viral loads of subsequent visits were followed. **Results:** Linear range for CAP/CTM v2.0 was confirmed (R2 0.9993). Standard deviation of replicates was 0.05 – 0.09 log10 copies/mL over the dynamic range. Analytic sensitivity showed 100% detection at 25 copies/ml and clinical specificity was 100%. Comparison of viral titer results from CAP/CTM v2.0 and CAP/CTM v1.0 yielded slope 1.07, intercept -0.19, R2 0.96, and mean bias (v2.0 – v1.0) +0.04 log10 copies/mL. Subanalysis of viral titers <200

copies/ml showed a mean bias of -0.19 log10 copies/mL. Eighteen of 25 low-positive CAP/CTM v1.0 patient samples had results available by both CAP/CTM v2.0 and m2000. Of these, 13/18 were positive by both assays, 1 negative by CAP/CTM v2.0, 3 negative by m2000, and 1 negative by both. Nine of the 25 patients were negative or < 48 copies/ml by CAP/CTM v1.0 during the follow-up period (1-12 months), 7 remained continuous or intermittent positive, and 3 had virologic failure (> 500 copies/ml). Six patients had no follow up data. **Conclusions:** The CAP/CTM v2.0 assay is an accurate and reliable test for HIV-1 viral load measurement with analytic performance comparable to CAP/CTM v1.0. The viral titers by CAP/CTM v2.0 for the clinically significant low positive range (<200 copies/ml) are lower when compared to CAP/CTM v1.0 but higher than m2000.

ID09. Multiplex Screening for Blood-Borne Viral, Bacterial, and Protozoan Parasites Using the OpenArray® Platform

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Introduction: The use of nucleic acid tests (NAT) for detection of pathogens has improved the safety of blood based products. However, as more emerging pathogens are recommended by the FDA for testing, there is an increased need for development of detection platforms testing multiple blood-borne pathogens including protozoan parasites. **Methods:** Our approach to this problem is the development of platform for detection of multiple blood-borne pathogens using a combination of two proven technologies: Taqman® chemistry for target identification and quantification, and OpenArray® for spatial multiplexing of target-specific assays. **Results:** The initial panel consisted of 56 newly designed and previously published TaqMan® assays for detection: 4 viral (HIV, HBC, HCV and WNV), 2 bacterial (Gram negative and Gram positive), and 3 protozoan parasites (*Leishmania donovani, Plasmodium falciparum,* and *Trypanosoma cruzi*). Each assay had been validated for specificity and detection efficiency for each purified DNA or RNA in the presence or absence of complex DNA background. The subset of validated assays (2-3 assays per individual target) was selected for a final panel and it was evaluated with pathogen spiked human blood and plasma samples. For most of the targets the limits of detection of tested organisms were comparable with existing real-time platforms and it was at the level of 500 copies/mL or higher. We achieved a sensitivity of detection of 500 copies/mL or lower when we incorporated a pre-amplification step with gene specific primers or used digital PCR for testing unamplified DNA or RNA samples. The final validation of the TaqMan® OpenArray® real-time PCR panel consisted of testing blinded human plasma or blood samples in the presence or absence of spiked pathogens. The testing of blinded samples produced no false-positive results and correctly identified pathogens present in samples with a limit of detection ranging from 10 cells/mL (*T. cruzi*) to 100-500 copies/mL for viral targets. **Conclusions:** These results indicate that the OpenArray® platform in combination with TaqMan® chemistry can provide a multiplex real-time PCR pathogen detection method and could potentially serve as the next-generation platform for infectious disease testing in blood.

ID10. Evaluation of Luminex Analyte Specific Reagents (ASRs) in a Multiplex PCR Format for the Detection of Multiple Diarrheal Pathogens

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Introduction: Gastrointestinal diseases presenting with identical symptoms can be caused by multiple infectious agents, which are diagnosed by sequential culture or assay methods that are time consuming and can result in inappropriate therapy. A rapid, multiplex assay capable of detecting these pathogens in a single PCR based assay would be clinically useful. **Methods:** We evaluated Luminex ASRs in a Multiplex PCR Format with a 20-plex primer mix to detect *E. coli* ST, LT, 0157, shiga toxin 1 and 2, *Salmonella spp., Shigella spp., Yersinia enterocolitica*, *Norovirus, Rotavirus, Adenovirus*, *C. difficile*, *Campylobacter spp.*, *Giardia, Cryptosporidium, Vibrio spp.,* and *E. histolytica* on the Luminex and MagPix systems after nucleic acid extraction on the EasyMag platform, using 300 positive frozen or fresh stool specimens and ATCC culture strains. Comparators were results from culture (*Salmonella* and *Shigella*), enzyme immunoassays [*Campylobacter* and Shiga-like toxin in *E. coli* (STEC)], DFA stains (*Giardia* and *Cryptosporidium*), direct microscopy (*Entamoeba* species) and PCR (*C. difficile, Norovirus*). Discrepant results were resolved by sequencing and alternate primer pairs. **Results:** Sensitivity and specificity after discrepancy analysis: *E. coli* 100% and 99.7% (culture/EIA 93.8% and 100%), *Salmonella* 94.4% and 99.6% (culture 94.4% and 99.7%), *Shigella* 100% and 99.3% (culture 85.7% and 100%), *Vibrio* 91% and 99.6%, *C. difficile* 93.9% and 98.9% (alternate PCR 91.2% and 99.2%), *Campylobacter* 100% and 99% (EIA 80% and 100%), *Giardia* 94.1% and 100% (DFA 100% and 99.7%), *Cryptosporidium* 99 and 100%, *Rotavirus* 90.9% and 99.3% (EIA 93.8% and 98.9%), *Norovirus* 94.4% and 98.6% (alternate PCR 81% and 95.7%). Of 12 samples identified as *E. histolytica*/dispar, only one was confirmed by both the Luminex and sequencing assays as *E. histolytica*. Total run time to final results was 5 hours. Limit of detection ranged from 300-2000 cfu for the bacterial pathogens. **Conclusions:** The Luminex ASRs for diarrheal pathogens are capable of reliably detecting a range of diarrheal agents in a multiplex format. Besides improving the specificity of detection, the assay identified co-infections with improved work flow efficiency.

ID11. Allele Frequency of an IL28B Treatment-Predictive SNP in HCV-Infected Patients

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Introduction: The likelihood of attaining a sustained virologic response in patients with HCV depends on viral-, disease-, and host related factors. Among host-related factors, the rs12979860 SNP, [CC genotype (vs CT/TT genotypes)], located 3 kb upstream of the IL28B gene is associated with an approximately 2- to 3-fold greater rate of sustained virologic response (SVR) in HCV genotype 1 infected individuals treated with combination pegylated IFN/ribavirin therapy. The favorable CC genotype has also been associated with a 3-fold increase in the rate of spontaneous clearance of HCV. In a recent US-based study, the favorable CC genotype was observed in 37% of Caucasians, 29% of Hispanics, and 14% of African Americans. We have validated an assay for the detection of IL28B SNP (rs12979860) by an allelic discrimination method, and have determined the allele frequencies in our predominantly Caucasian HCV population. **Methods:** We genotyped 112 random HCV-infected patients referred to our lab for HCV viral load monitoring. Human DNA was amplified using primers and TaqMan probes targeting the IL28B rs12979860 SNP region. Genetic variants were identified by an allelic discrimination method using SDS software (Applied Biosystems ABI 7500). The assay was validated in comparison with a commercially available reagent kit (LightMix Kit IL28B, rs12979860) that uses melting curve analysis to distinguish rs12979860 genotypes. **Results:** Among the 112 patients tested, 101 were Caucasian, 4 African American, 3 Asian, 2 American Indian, and 2 had unknown ethnicity. In the Caucasians, the IL28B genotype frequencies showed 34 CC (34%), 59 CT (58%), and 8 TT (8%) genotypes. The 34% CC favorable genotype (95% CI, 25% to 43%) in Caucasians was not significantly different than the 37% CC frequency reported in the literature (95% CI, 34.5% to 40%)(P=0.52). There was 100% concordance in IL28B genotypes between our TaqMan assay and the LightMix assay in all 50 samples with dual data. We also performed a blinded sample exchange of 22 DNA samples (comprising 6CC, 12CT, and 4TT genotype) with 2 different labs, and found 100% genotype concordance. Additionally, 6 samples were analyzed by Pyrosequencing, which confirmed the expected genotype. **Conclusions:** The IL28B TaqMan genotyping assay is technically robust and accurate, and has prognostic and treatment implications in patients infected with HCV, particularly hepatitis C, genotype 1.

ID12. Genotyping of IL28B Polymorphism rs12979860 by High Resolution Melting of Unlabeled Probe

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Introduction: The single nucleotide polymorphism (SNP) rs12979860 maps 3 kb upstream of the human interleukin 28B (*IL28B*) gene on chromosome 19, which encodes the type III interferon IFN-gamma3. Genotyping of the SNP, which comprises a C or T dimorphism, is clinically useful in individuals infected with HCV genotype 1 virus (HCV-1). Treatment of chronic HCV infection generally consists of a combination of PEG-Interferon-alpha-2a or 2b and ribavirin. Genome-wide association studies have demonstrated the C/C variant is associated with an improved response and spontaneous clearance to the drug regimen, and its identification may allow a more individualized therapy and an improved sustained virological response. An unlabeled probe-based high resolution melting (HRM) real-time PCR has been developed on the LightScanner 32® (LS 32) (Idaho Technology Inc., UT, USA), to serve as a rapid and inexpensive method to identify the rs12979860 polymorphism in patients infected with HCV-1. **Methods:** An unlabeled probe with a C3 blocker (Suprenom, Singapore) was designed to perfectly match the C allele of rs12979860. Real-time PCR amplification was performed with a ratio of 1:5:5 of forward primer, reverse primer and unlabeled probe in the LightScanner® Master Mix with incorporated LC Green® PLUS (Idaho Tech) on the LS 32, followed by a final denaturing/ re-annealing step to generate HRM profiles. The region of the unlabeled probe melt was manually selected and the fluorescence, normalized. The LS 32® software (v1.0.0.33) was used for genotype analysis, clustering and auto-grouping of the unknown samples. Forty archived nucleic acid extracts retrieved from previously processed HCV-1a and -1b infected patient plasma, were analyzed. The HRM results were validated using a co-developed sequencing-based method. **Results:** HRM generated unique melting profiles for all three (C/C, C/T, T/T) variants. Melt peaks for the unlabeled probe with a mismatched base (for T allele) and a perfectly matched base (for C allele) were clearly discernable

at 64°C and 72°C, respectively. Normalized melt curves reproducibly showed distinct patterns for homozygous (C/C and T/T) and heterozygous (C/T) genotypes. Of the 40 clinical samples tested, 27 (67.5%) had C/C; 11 (27.5%), C/T; and 2 (5%), T/T genotypes. **Conclusions:** The above rapid and low-cost in-house real-time PCR assay was able to genotype the clinically important *IL28B* polymorphism in HCV-1 infected patients, and provides a good alternative to Sanger sequencing. The allelic frequencies may account for a difference in treatment response in different ethnic groups; this aspect will be further investigated in our local multi-ethnic population.

ID13. New HIV-1 SuperLow Assay for Viral Load Monitoring

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Introduction: As new antiretroviral therapies (ART) such as Integrase inhibitors become standard of care, accurate ultra low viral load monitoring will become increasingly important. Equally important will be clinical monitoring of ART potency using assays that detect below the current FDA approved assay cutoffs. Here we describe a new HIV-1 SuperLow assay for detection of HIV-1 RNA at ultra low levels, developed using a modified protocol of a CE marked commercial kit. **Methods:** Viral subtype B RNA was extracted on bioMerieux's EasyMAG platform and analyzed using a proprietary HIV-1 SuperLow Assay that utilizes components of bioMerieux's commercially available (RUO) EasyQ HIV-1 v2.0. For Linearity determination, Virology Quality Assurance (VQA) stock material at 10^7 was diluted 1:10 serially 5X in normal HIV-1 negative human plasma to yield dilutions of 1:10, 1:100, 1:1000, 1:10,000, 1:100,000. The Limit of Detection (LOD) was determined by analyzing dilutions of VQA viral standard made in HIV-1 negative human plasma. At least 27 replicates of each dilution were utilized. Probit analysis was performed on HIV-1 replicate dilution series 3, 6, 12, 24, 48, 72, 96 c/mL to determine the 95% Hit Rate using Percent Detected (PD) values at each dilution. Excel 2007 (Microsoft) function NORMSINV(z) was used to translate PD to probit values. **Results:** Linearity determination, testing diluted samples from 10^2 - 10^7 c/mL, demonstrated a proportional relationship between the dilution factor and number of HIV-1 RNA copies reported by the assay. The LOD was determined by testing replicates of normal human plasma spiked with a range of HIV-1 RNA from 3 to 96 c/mL. Probit analysis revealed that the concentration of HIV-1 RNA detected with 95% probability was 15 c/mL (1.18 log c/mL). **Conclusions:** In this data set analysis, a new HIV-1 SuperLow assay demonstrated good linearity and large dynamic range from 3 to $8.3x10^6$ c/mL. Probit analysis from a HIV-1 dilution series of subtype B specimen demonstrated an impressive 95% hit rate at 15 c/mL. The HIV-1 SuperLow assay will be a valuable tool for monitoring HIV-1 viral load in drug development and clinical trial programs.

ID14. Genotyping of the Hepatitis C Virus on the eSensor® XT-8 System

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Introduction: Hepatitis C virus (HCV) is a leading cause of chronic liver disease in the United States. The prevalence of HCV infection has been estimated to be 1.8% in the general population, resulting in an estimated 2.7 million cases of chronic HCV infection in the United States. The genotype is helpful in defining the epidemiology of Hepatitis C. More importantly, knowing the genotype of HCV is helpful in making recommendations and counseling regarding therapy. The eSensor® HCV Genotyping Test is a new assay designed to genotype a panel of 8 HCV sub-types: 1a, 1b, 2ac, 2b, 3, 4, 5 and 6ab. The end product of the COBAS® Taqman® HCV test (Roche Molecular Diagnostics) serves as the template for a nested PCR followed by a direct analysis on the electrochemical eSensor® XT-8 detection system. **Methods:** This study was designed to determine the specificity of the eSensor® HCV Genotyping assay compared to sequencing the end product of the COBAS® Taqman® HCV test in the 5' untranslated region. Uniplex primers were designed to amplify conserved viral target regions for Hepatitis C subtypes 1a, 1b, 2ac, 2b, 3, 4, 5 and 6ab that contained hybridization sites for the XT-8 capture probes and signal probes. For eSensor® XT-8 analysis, amplified samples were added to hybridization buffer containing ferrocene-labeled signal probes and then hybridized to electrode-bound capture probes in the eSensor® XT-8 cartridge. Hepatitis C genotypes were detected by measurement of the current produced at each electrode during alternating current voltammetry. **Results:** The limit of detection for genotyping the HCV ranged from 25 IU to 6.9 E8 IU of the input template viral load. We tested 344 samples and achieved a combined agreement of 99.4% with zero no calls. Sequencing of the 5' untranslated region was the method comparator assay. The overall assay time from sample to result is ~1.8 hours with ~20 minutes hands-on time. **Conclusions:** The eSensor® HCV Genotyping Test successfully genotypes sub-types 1a, 1b, 2ac, 2b, 3, 4, 5, 6ab and mixed genotypes on the eSensor® XT-8 cartridge. This eSensor® product is under development and is not available for sale in the United States.

ID15. Evaluation of 3 Commercial Molecular Assays for the Detection of *Clostridium difficile* in Comparison with Rapid EIA and Toxigenic Culture

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Introduction: *Clostridium difficile* infection (CDI) is the most common cause of health care-associated diarrhea. Enzyme immunoassays (EIAs) for glutamate dehydrogenase (GDH) and toxin A/B are most commonly used to diagnose CDI but lack specificity and sensitivity respectively. This study aims to compare the performance of three commercial nucleic acid amplification assays against conventional rapid EIA and toxigenic culture. **Methods:** Forty-nine stool samples from patients with suspected CDI were used for the study. The samples were previously tested for GDH and toxins A and B by TechLab C. diff Quik Chek Complete® EIA method. The three molecular assays in the evaluation were the Cepheid Xpert, TibMolBiol LightMix and Meridian illumigene *C. difficile* assays. The first 2 assays are based on real-time PCR while the illumigene assay uses loop-mediated isothermal amplification (LAMP) technology. For the Xpert and illumigene systems, stool samples were used directly in the assays and these systems performed the entire procedure from DNA extraction to result analysis. For the TibMolBiol assay, DNA was extracted from stool samples using the BioMerieux NucliSens easyMAG automated extraction system. Amplification and detection were performed on the Lightcycler version 2.0. All stool samples that were GDH positive and toxin negative by EIA were sent for *C. difficile* toxigenic culture. **Results:** Using the combined results of toxigenic culture, GDH and toxin EIA as the reference method, there were 30 positive and 19 negative toxigenic *C. difficile* samples in the study. All 3 molecular assays gave similar performance results. The sensitivities for the LightMix, Xpert and illumigene assays were 100%, 100% and 92.9% respectively, while the specificities for the LightMix, Xpert and illumigene assays were 100%, 94.7% and 100% respectively. The hands-on and turnaround time was shortest for the Xpert assay and longest with the LightMix assay. For the illumigene assay, 7 (15%) samples had to be repeated due to initial invalid results. **Conclusions:** Molecular assays prove to be sensitive, specific and rapid for the detection of toxigenic *C. difficile* and are an attractive alternative to the current methods for the diagnosis of *C. difficile* infection.

ID16. Laboratory Evaluation of the Idaho Technology BCID FilmArray

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Introduction: The detection of microorganisms in blood samples has been improved with automation of the blood culture systems, however, identification of the agents still requires culture based methods that can take several days. Faster identification of limited types of organisms is possible through FISH methods and a few organism targeted PCR assays. The ability to identify a large number of the most common pathogens would improve patient management by allowing greater ability to target antimicrobial therapy. The Idaho Technology FilmArray Blood Culture Identification (BCID) system is a multiplex PCR assay that targets 19 bacteria, 5 yeasts, and 4 antimicrobial resistance genes. This assay is able to provide results in approximately 1 hour from the time a positive blood culture is flagged by the instrument. **Methods:** Blood was collected into BactAlert FAN (Biomerieux) bottles per standard of care and incubated in the BactAlert instrument. When a bottle was flagged as positive it was processed for Gram stain and subculture. Next a 250 µL sample was collected by syringe, added to the BCID FilmArray diluent and transferred to the sample port of the pouch. The pouch reagents were rehydrated using the provided reagent solution. The pouch was scanned by the instrument and placed in the FilmArray processor. Sample information was entered and the assay run was started. The instrument performed the extraction and nested PCR on board. **Results:** A total of 99 positive blood cultures have been processed to date. The FilmArray correctly identified the pathogen in 83 (84%). The FilmArray did not detect probable pathogens in 13 (13%) samples however all of these were organisms not included in the panel. The only misidentifications (3) in which no other agents were identified occurred with *Enterobacter cloacae*, *Acinetobacter spp.,* and *Pseudomonas spp.*, which are known to be problems with this first generation system due to contaminants in the pouch assembly process. Later generation pouches were prepared from a different supplier of plastic and addressed this problem. **Conclusions:** The BCID FilmArray was able to detect 100% of organisms included in the panel and 100% of the cases with antimicrobial resistance factors that were included in the panel within 1 hour. Once available for routine testing this system promises to provide significant improvement in managing patients with bloodstream infections.

ID17. Comparison of a Rapid Lysis Method to Automated DNA Extraction for HSV and VZV PCR Detection from Ocular and Oral/Nasal Swab Specimens

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Introduction: We process dermal and genital swabs received for Herpes Simplex Virus (HSV) and Varicella-Zoster Virus (VZV) PCR by a rapid cell lysis method that is faster and provides greater sensitivity than automated DNA extraction. Swabs from ocular and oral/nasal sources are also received for HSV and VZV PCR. These sources are collected in M4 Viral Transport Media; therefore requiring DNA extraction. The aim of this study was to compare the lysis method to MagNA Pure extraction for the detection of HSV and VZV DNA from ocular and oral/nasal swabs. **Methods:** Two-hundred forty-five swab specimens (76 ocular, and 169 oral/nasal) from patients suspected of having either HSV or VZV infection were processed by the rapid lysis method and the MagNA Pure extraction method. Dual rayon swabs were inoculated with patient specimen; one swab was placed in 3 mL of M4 media, followed by extraction of 200 µl on the MagNA Pure instrument and eluted into 100 µl. The second swab was placed into a tube containing 600 µl of neutralization buffer (NB), followed by heating/shaking at 99° C and 1400 rpm for 6 minutes. Five µl of each preparation were then assayed for the presence of HSV and VZV DNA by real-time PCR on a LightCycler 2.0. **Results:** Of 245 specimens assayed, HSV DNA was detected in 44 (18%) and 60 (24%) specimens processed by the MagNA Pure and the lysis technique respectively. The 16 discrepant samples were originally positive. The specimens processed by the cell lysis method were detected on average 4 cycles (Cp) sooner than the extracted specimens. No inhibition was detected based on the presence of an internal control. VZV DNA was detected in 4 of 245 specimens (1.6%) processed by both methods. The Cp values were roughly equivalent. **Conclusions:** The lysis method is faster than automated extraction on the MagNA Pure instrument, and provides equal or increased sensitivity for detection of HSV/VZV DNA by PCR from ocular and oral/nasal swabs.

ID18. *Mycobacterium smegmatis* Was Frequently Detected in Skin Biopsies from Patients with Cutaneous Infectious Granuloma

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Introduction: Cutaneous infectious granuloma (CIG) is a common soft-tissue infection and mycobacterial infection has been considered an etiology. MycoID, a molecular system combining broad-range PCR amplification and suspension array identification, was developed to detect and differentiate 17 medically important mycobacterium species. We applied the MycoID system to detect and identify Mycobacterium in skin biopsy specimens from patients with CIG. **Methods:** A total of 119 skin biopsy tissue specimens were collected from patients (100 children and 19 adults) between 2001 and 2010 from Beijing. Among them, 72 were collected from patients with clinically and histologically confirmed CIG and 47 from patients with reactive skin rash (erythema nodosum, n=38; erythema induratum, n=9) as controls. DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) biopsies by using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA). A real-time PCR human β-actin assay was used as the internal quality control of extracted DNA. The MycoID system was performed to detect all mycobacterium species with the capacity to identify 17 medically important species. **Results:** Mycobacterial DNA was detected in 30 (41.7%) of 72 CIG and 10 (21.3%) of 47 controls (OR=2.64, P=0.021). Mycobacterial species detected in CIG included *M. intracellulare* (1), *M. tuberculosis* (3), *M. smegmatis* (13), *M. szulgai* (1), *M. kansasii* (1) and other species (11). In contrast, mycobacterial species detected in the controls included *M. tuberculosis* (1), *M. smegmatis* (2), *M. kansasii* (1) and other species (6). *M. smegmatis* was detected with significantly higher frequencies in CIG (18.1%) than in the controls (4.3%, OR=4.92, P=0.027). **Conclusions:** Mycobacterial species, especially *M. smegmatis*, were detected at higher frequencies in biopsies from patients with CIG. The MycoID may provide an alternative tool for the diagnosis of mycobacterial infections in FFPE skin tissues.

ID19. Derivation of Virtual Morphology, E6-E7 HPV Quantification and Cell Cycle Analysis Using Liquid Based Pap Samples Defines the Biology of ASCUS Samples

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Introduction: Current practice using liquid based Pap samples involves manual/image analysis for cell morphology, addition of biomarkers to measure cell proliferation and extraction of nucleic acids to determine HPV status. Recently, with the release of 3Dx™ technology, it is possible to derive all of the above on the same cell in suspension. In a test of feasibility, we analyzed 25 liquid based samples on the iCyt Eclipse Analyzer using its electronic volume capabilities in an attempt to derive virtual morphology of the cell populations on a system capable of high throughput. **Methods:** The samples were stained following published procedures with a cocktail of anti-sense oligonucleotides to E6,E7 of HPV and DAPI at a concentration of 1 µg/ml after the last cell wash. The DNA dye was allowed to stain for 30 minutes at room temperature before collection on the Eclipse Analyzer. **Results:** The inclusion of the DNA dye allows an immediate assessment of the submitted sample for the presence of intact cells and our analysis revealed two samples that would necessitate resubmission due to a complete lack of events containing a nucleus. Of the remaining 23 samples, combinations of the nuclear dye along with cellular complexity and electronic volume allowed the derivation of cellular debris, enucleated and nucleated ectocervical cells along with multinucleated forms and polymorphonuclear leukocytes. The increasing volume of the differing ectocervical cell populations creates a virtual N/C ratio, and as expected this population exhibited an increased proliferative rate matching a dysplastic cytology. In a subset of the samples (n=8) all defined as ASCUS with HPV+ DNA, the E6,E7 positive samples (n=3) contained fewer nucleated ectocervical cells with a gross increase in PMNS and a moderate S-phase; the negative samples (n=5) exhibited an increase in high N/C ectocervical cells with an increase in S-phase within a normal PMN background. This data suggests an ability to define whether ASCUS samples are normal (NILM) or LSIL/HSIL within the clinical designation of ASCUS. **Conclusions:** The combination of the 3Dx™ technology collected on the iCyt Eclipse Analyzer revealed the ability to derive virtual morphology of liquid based Pap samples while obtaining the additional capabilities of sample acceptance along with cell cycle analysis. Following this successful feasibility study, additional studies are underway to determine whether the ASCUS cytologic diagnosis can be eliminated.

ID20. Development and Validation of a Comprehensive Vaginitis Diagnostic Panel

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Introduction: Vaginitis is caused by several often misdiagnosed infections with an incidence rate of 12-13 million new cases per year. The three most common types of vaginitis are trichomoniasis (*Trichomonas vaginalis*), bacterial vaginosis (*Gardnerella vaginalis* and other opportunistic anaerobic bacteria), and candidal vulvovaginitis (*Candida spp*.) and account for 90% of vaginitis cases. Typical detection methods of vaginitis include wet mount to visualize clue cells, Gram-stain, culture, pH testing, and potassium hydroxide treatment. While these tests are an indication of the presence of vaginitis, they often do not determine the specific pathogen responsible for the symptoms. Initial treatment of vaginitis is often not successful and 50% to 80% of patients experience recurrence of vaginitis within 1 year. We have developed a series of simple, high-throughput tests to better characterize the presence, virulence, and concentration of organisms associated with vaginitis, as well as the ability to monitor the level of natural flora. **Methods:** The vaginitis diagnostic panel is a laboratory developed test utilizing multiplex PCR and automated capillary electrophoresis to detect the presence of the three most common causes of vaginosis. Workflow management and data upload, presentation, and preliminary analysis were performed using TeleGene® software. Clinical cutoffs based on literature values were established above the limit of detection of each analyte. **Results:** *T. vaginalis*, *G. vaginalis*, eight *Candida spp*., and an internal control marker were able to be differentiated from each other using capillary electrophoresis. Analytic Limit of Detection was 24 copies/mL for *T. vaginalis*, $1.96x10^3$ copies/mL for *G. vaginalis*, and $9.8x10^2$ copies/mL for the Candida species. Of 112 species tested for cross-reactivity, only *Bifidiobacterium spp*. above 10^5 copies/reaction had cross-reactivity with the *G. vaginalis* primer set. Detection rates matched literature values of 7% for *T. vaginalis*, 50% for *G. vaginalis*, and 20% for *Candida spp*. **Conclusions:** The vaginitis panel is a fast, reliable, sensitive, and cost-effective way to determine the causative agent of vaginitis. Reflex tests to determine the *Candida* species present, determine the presence of other organisms associated with vaginitis, and to determine the ratio of *Lactobacillus* bacteria to anaerobic bacteria will be developed to generate additional information. This panel of tests will allow doctors to more accurately determine the organism responsible for vaginitis, allow for better initial treatment of the infection, and provide a means to measure treatment effectiveness.

ID21. Evaluation of the EraGen Multicode®-RTx PCR Assay for the Quantitative Detection of BK Virus in Urine and Plasma Specimens

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Introduction: Primary infection by BK polyomavirus (BKV) is typically asymptomatic and followed by viral latency, mainly in the urogenital tract. Reactivation of the latent virus may occur in immunocompromised patients and is a particular threat to renal transplant patients. Quantitative detection of BKV DNA in both the urine and in the blood is becoming increasingly important as an early marker for polyomavirus-associated nephropathy. The EraGen Multicode®-RTx PCR assay, which uses a novel

Multicode chemistry, provides a quantitation BKV PCR assay that is adaptable to multiple DNA extraction, amplification, and detection platforms. **Methods:** BKV DNA was extracted from urine (n=38) and plasma (n=18) specimens using the automated NucliSENS easyMAG instrument and reagents (BioMerieux). Quantitative PCR analysis was performed using Multicode®-RTx PCR analyte specific reagents (EraGen Biosciences) and the Lightcycler 2.0 (Roche) for amplification and detection. **Results:** The linearity and precision of the EraGen assay, determined by analysis of AcroMetrix OptiQuant BKV quantification panel members at 5 different virus titers, demonstrated a linear response from 500 (Log10 = 2.7) cp/mL to 5 x 10^6 (Log10 = 6.7) cp/mL. This assay was repeated at least 5 times (CV < 2.4). Linear regression analysis of the panel values produced a coefficient of determination of 0.996. This data indicates a high degree of precision and accuracy for the assay. The limit of detection (LOD), using Optiquant panel BKV diluted in BKV-free urine, was 200 cp/mL. 56 clinical specimens (38 urine and 18 plasma) were analyzed by the EraGen assay. BKV was detected in 29 of 32 BKV positive specimens and in 3 of 24 BKV negative specimens. Based on these results, the sensitivity and specificity of the assay was 90.6% and 87.5%, respectively. Linear regression analysis, performed on results from 23 BKV positive urine specimens, produced a slope of 0.897 and a coefficient of determination of 0.881. These results indicate consistent agreement between reference and observed values throughout the range of viral load levels tested. **Conclusions:** Quantification of BKV using an easyMAG extractor, EraGen reagents and Lightcycler thermocycler, was rapid and sensitive, and performed comparably to reference assays using both urine and plasma clinical samples. This assay will be useful for monitoring BKV load in transplant recipients.

ID22. Comparison of Two Pyrosequencing Platforms for Bacterial Pathogen Identification

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Introduction: Pyrosequencing of the variable regions of the 16S rDNA gene is a rapid and effective strategy for identification of bacterial pathogens. In 2003, the Texas Children's Hospital (TCH) Molecular Microbiology Laboratory (MML) implemented pyrosequencing using the PSQ 96MA (Qiagen, Inc.) platform for the purpose of identifying organisms that produced inconclusive results via conventional microbiological techniques (morphology, biochemical testing, etc.). Recently, a new pyrosequencing platform, the PyroMark Q24 (Qiagen, Inc.), was introduced, offering improvements in the user interface as well as improvements in instrument capabilities. Instrument characteristics and sequencing data generated by the PSQ 96MA and the Q24 were compared to evaluate the performance of the Q24. **Methods:** From January to December 2010, a total of 90 diverse bacterial isolates were submitted to the TCH MML for molecular identification by pyrosequencing. Bacterial DNA from all 90 isolates was extracted and amplified separately using primers previously designed to target the V1, V3, and V6 variable regions of the 16S rDNA gene. Following amplification, samples were pyrosequenced with both the PSQ 96MA and the Q24 instruments. Characteristics specific to pyrosequencing were compared for each platform and categorized into three main groups: instrumentation (number of reaction wells, detection cameras, nucleotide cycle dispensations, total pyrosequencing run time, and sequence lengths), sample preparation (reagent and sample volumes, total set-up time), and computer/ software requirements. **Results:** Additional detection cameras on the Q24 provided the opportunity to increase the number of nucleotide cycle dispensations resulting in increased run times and sequence lengths when compared to the PSQ 96MA. Additionally, while the Q24 only had the capacity to sequence 24 samples in one run, the sample preparation chemistry required smaller reagent and sample volumes. Amplicon sizes ranged from 105bp to 192bp while read lengths averaged 34 bases per region for the PSQ 96MA and 51 bases per region for the Q24. Also, the Q24 is approximately half the size of the PSQ 96MA and requires only a USB flash drive to transfer run specifications and data; compared to the additional computer connection required for PSQ 96MA management. **Conclusions:** Compared to the PSQ 96MA, the Q24 provided longer sequences with reduced reagent and sample volumes; however, the Q24 required a longer pyrosequencing run time and had fewer reaction wells per run. Overall, reagent optimization, improvements in software, and compact instrument size make the Q24 an attractive pyrosequencing platform for implementation in a molecular diagnostics laboratory.

ID23. Comparison of Assay Performance Between Cell Culture and a Rapid Automated Molecular Test for HSV Typing of Clinical Samples

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Introduction: Laboratory confirmation of presumptive Herpes Simplex Virus (HSV) infection historically involves manual cell culture often exceeding 24 hour time to result. Molecular methods have been known to increase the positivity rate by 20% to 25%. An automated, rapid molecular diagnostic system may improve the availability, accuracy, and time to result. The investigational GenturaDx IDbox™ HSV 1/2 assay is a real-time PCR test for rapid and qualitative detection and discrimination of Herpes Simplex Virus type 1 (HSV1) and 2 (HSV2) from lesion specimens in approximately 3 hours. The IDbox system integrates nucleic acid extraction, purification, PCR amplification, and detection using disposable cassettes preloaded with specific reagents to minimize hands-on time, risk of errors, and contamination. The first data using clinical specimens was obtained in 2011 comparing the IDbox HSV 1/2 assay and the Diagnostic Hybrids ELVIS® HSV test kit at a clinical laboratory. **Methods:** Seventy-nine clinical specimens submitted for HSV testing were evaluated at an independent clinical laboratory with the GenturaDx IDbox HSV 1/2 Assay and the ELVIS HSV test kit. Discordant results were resolved by sending aliquots of those samples to a national clinical laboratory for molecular testing using the Roche LightCycler® HSV 1/2 Detection Kit for discrepancy analysis. **Results:** Of the 79 clinical specimens, 56 were from the vaginal region: 22 from the vagina and 34 from the surrounding area. The remaining 23 samples were from various body sites (throat, face, male specific for example). Of the 79 specimens, the IDbox HSV 1/2 detected 20 more positive samples than the ELVIS assay, 12 of which were confirmed positive by the Roche ASR molecular method. The positivity rate was 59% for the IDbox and 37% for the ELVIS test. Fifty of 79 (63%) specimens had concordant results between the IDbox HSV 1/2 and ELVIS HSV tests. After discrepancy analysis, the overall concordance percentage increased to 86%. For the 56 vaginal region specimens alone the concordance rate after discrepancy analysis was 86%. For the 22 vaginal specific specimens the concordance rate was 91%. **Conclusions:** This study demonstrates that the GenturaDx IDbox HSV 1/2 assay has the potential to provide faster and more sensitive results than cell culture tests for the diagnosis of HSV 1 and 2 infections.

ID24. Design and Performance of Adenovirus, VZV, HSV-1, and HSV-2 Quality Control Materials for Assessing and Monitoring the Performance of Molecular Assays

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Introduction: Adenovirus, VZV, HSV-1, and HSV-2 molecular diagnostics utilize sample types such as cerebral spinal fluid (CSF) or plasma. Molecular assays that monitor viral load from transplant recipients typically utilize plasma based specimens and require calibrators to standardize the results. Regulations and guidance documents recommends the use of full process controls in similar matrices as patient specimens for validating/verifying assays as well for external quality control. In order to assist laboratories in meeting testing guidelines for plasma and CSF based Adenovirus, VZV, HSV-1, and HSV-2 testing, a quantification panel and high and low positive control were developed. **Methods:** Accelerated stability of the product was assessed using the QIAGEN Artus® VZV PCR ASR and an in-house developed HSV and Adenovirus assay and indicated that the product is stable for 18 months at -20°C. The performance of the plasma panel, which contains the 1E6, 1E5, 1E4, 1E3, and 1E2 copies/mL panel members, was assessed on several platforms with varying extraction, detection, and reagents showed a limit of detection at the 1E2 copies/mL level for the assays tested. **Results:** A reproducibility study was conducted with the High and Low Controls with the resulting intra-run reproducibility maximum of 35% CV and an inter-run reproducibility maximum of 35%. 11 laboratories evaluated the linearity, reproducibility, and LOD of the quality control materials on various quantitative and qualitative assay platforms. **Conclusions:** The performance and stability of Adenovirus, VZV, HSV-1, and HSV-2 quality control materials indicates that they are useful tools to assess the performance characteristics of molecular assays as well as for use as a daily external run control.

ID25. Electronic Medical Record-Based Syndromic Surveillance and Molecular Testing Data to Track Influenza Activity in a Community

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Introduction: One indicator of influenza prevalence in the community is the frequency of symptoms such as “fever” plus “cough” reported by patients and/or recorded by their attending physicians in visit notes, available through “syndromic surveillance” systems. We compare the frequency of influenza-like illness (ILI) symptoms among patients seen at NorthShore University HealthSystem over a two-year span as collected by an electronic medical record (EMR)-based syndromic surveillance system with the results of molecular testing for influenza. **Methods:** 847,728 primary care outpatient encounters were analyzed for EMR evidence of ILI. %ILI is defined as the percentage of these visits where fever and respiratory findings were recorded. The weekly %ILI, total molecular influenza tests, and positive influenza results were recorded between February 2009 and April 2011, and a temporal analysis was performed. Correlation

coefficients were calculated using Microsoft Excel. **Results:** Generally, %ILI tracked well with the number of total influenza specimens, the number of positive influenza tests and the percentage of influenza tests that were positive (correlation coefficients 0.77, 0.78, 0.83), with some notable exceptions: 1) A surge of both %ILI and total influenza testing occurred immediately after news reports of the pandemic outbreak in Mexico. This surge was not correlated to positive test results, and occurred days before a second wave of reported ILI and molecular testing including increased positive results, corresponding to the true spread of pandemic influenza A in the Chicago area. 2) While the timing of peak ILI activity and peak testing activity were closely associated in all studied "flu seasons", the quantity of testing was much greater during pandemic periods than non-pandemic periods despite similar ILI activity in pandemic and non-pandemic periods, suggesting that testing intensity does not predict disease intensity. 3) Several smaller spikes in %ILI occurred in December 2009 and February 2010 with no corresponding increase in total influenza testing or positive tests, indicating an interpretation by the physicians that testing for influenza was unnecessary at that time or that this represented another viral disease in the community with similar signs and symptoms as influenza. **Conclusions:** Although generally comparable, syndromic surveillance differs in important ways from laboratory testing data, preventing the substitution of one for the other. Used together they present a good picture of influenza activity. When %ILI rises and confirmed influenza does not, it suggests a non-detected viral illness is present in the community.

ID26. Evaluation of Focus Diagnostics Simplexa™ HSV 1 & 2 Direct Sample-to-Answer Real-time PCR Assay

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Introduction: The high prevalence of herpes simplex virus (HSV) infections makes it a significant public health concern. Prompt detection and differentiation of HSV infections can assist in patient management, and is especially important in cases of HSV meningoencephalitis. The Simplexa™ HSV 1 & 2 Direct assay is in development as a sample-to-answer multi-analyte detection system performed on the 3M Integrated Cycler instrument. Swabs in viral transport media and CSF specimens are loaded directly onto a Direct Amplification Disc without extraction or other specimen preparation. The goal of this study was to compare the performance of the Simplexa™ HSV 1 & 2 Direct assay relative to culture and to real-time PCR methods that require extracted specimens. **Methods:** Limit of detection (LoD) studies were performed to determine the analytical sensitivity of the assay. Inhibition, interference and cross reactivity were evaluated using a panel of bacteria, viruses and potentially interfering substances. Relative sensitivity and specificity were determined by testing blind panels of de-identified patient specimens and comparing results with those obtained using culture or a real-time PCR method that necessitate prior nucleic acid extraction. **Results:** The LoD studies showed that the Simplexa Direct assay detected HSV-1 strains at less than 40 TCID50/mL and HSV-2 strains at less than 5 TCID50/mL. No inhibition, interference or cross reactivity was observed. Relative sensitivity and specificity for swab samples were 100% (42/42) and 97.1% (102/105) for HSV-1, and 100% (40/40) and 100% (107/107) for HSV-2 samples. Relative sensitivity and specificity for CSF samples were 100% (36/36) and 97.7% (94/96) for HSV-1, and 94.6% (35/37) and 98.9% (94/95) for HSV-2 samples. **Conclusions:** The Simplexa™ HSV 1 & 2 Direct assay was capable of directly detecting and differentiating HSV-1 and HSV-2 from un-extracted clinical specimens with performance comparable to real-time PCR method that uses upfront nucleic acid extraction. The assay and instrumentation provide a compact system for rapid (~1 hour) detection of HSV-1 and HSV-2 directly from swab and CSF samples.

ID27. High-Throughput Direct Detection of *Clostridium difficile* from Stool Specimens Using the Simplexa™ *C. difficile* Universal Direct Assay

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Introduction: The increasing prevalence of *C. difficile*-related disease has resulted in the need for accurate and rapid diagnostic methods. Detection of toxin-producing organisms in stool samples is now routinely performed using PCR methodologies. However, high-throughput testing options are currently unavailable. To address this need, we have developed the Simplexa™ *C. difficile* Universal Direct Assay. This assay can directly detect organisms in stool without nucleic acid isolation procedures, and has a high throughput capacity (up to 96 samples tested in <2 hours). **Methods:** Fresh stool samples (n=189) were transferred into dilution buffer and incubated at 97°C for 10 minutes on a heating block. Samples were then applied directly to a 96 well Universal Disc, along with the reaction mix, and real-time PCR was carried out using the 3M™ Integrated Cycler. Assay performance was determined by performing analytical limit of detection (LoD) and reproducibility studies. Results were compared with those of 3 other methods: 1) a method involving a complete nucleic acid extraction step; 2) culture; and 3) a commercially available PCR assay. The effect of potential interfering substances was assessed by adding relevant concentrations of these substances to samples prior to testing. **Results:** The Simplexa™ *C. difficile* Universal Direct assay was at least as sensitive as the comparator methods, while providing greater throughput and more rapid test results. The LoD of the assay was 0.04 CFU/reaction. There was no interference from any of the 20 potential interfering substances tested, including blood, antibiotics, and diarrhea remedies. Inter- and intra-assay reproducibility assays yielded CVs of <2%. Results from a single specimen could be obtained in approximately 70 minutes, and up to 96 samples could be processed in <2 hours. **Conclusions:** The performance of the Simplexa™ *C. difficile* Universal Direct assay was comparable to the other methods. With no requirement for nucleic acid extraction, the assay provides significantly reduced turnaround time without compromising detection sensitivity.

ID28. Evaluation of a Molecular Diagnostic Method for the Clinical Detection of Hepatitis B Virus

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Introduction: During the past few years, pathogen detection by molecular diagnostic methods has become a staple in countless clinical laboratories. Yet, as usage and implementation of the foundational technology expands, the fundamental rigidity with current vendor solutions has yet to be solved. Here we develop a novel molecular diagnostic workflow that offers flexibility in sample type input while preserving throughput and performance. **Methods:** HBV-specific primers and fluorescent probes were designed from Hepatitis B virus Surface Antigen S gene by bioinformatics analysis. The primer and probe set for the internal positive control (IPC) DNA were designed from a Tobacco Mosaic Virus movement protein gene that does not exhibit any cross-reactivity toward the target gene. The optimization procedures of real-time PCR assay were performed using DNA template synthesized de novo. In order to improve specificity, chemical hot-start method using specific enzyme instead of antibody using in conventional hot-start method was developed. For convenience of use, all components required for PCR reaction, including thermo stable DNA polymerase, dNTP, as well as primers and probes, were aliquoted for one reaction per tube and lyophilized. We evaluated the sensitivity and specificity with a standard control for calculating the limit of detection, HBV genotype performance panel (PHD201), and 68 clinical samples. **Results:** Basic performance metrics of the Hepatitis B Virus kit were analyzed. The limit of detection was 50 IU/mL, and dynamic range was $5X10^1$ IU/mL to $5X10^{10}$ IU/mL. The coefficient of variation in the dynamic range was <2%(Ct value). Comparison analysis against Roche TaqMan96 HBV system showed 0.9272(R2) concordances. To evaluate the method effectiveness and throughput, published data of current vendor platforms were analyzed and compared against actual workflow measurements. Analysis revealed that processing 48 samples of a single sample type took on average 4.17 hours with the ExiStation platform, compared to calculated times of 8.45 and 6.67 hours for Roche and Abbott platforms, respectively. Varying input sample types (serum, plasma) did not add additional time in ExiStation processing a total of 48 samples. Additionally, processing 96 samples took only 5.25 **Conclusions:** Our novel molecular diagnostic workflow, ExiStation, has overcome the input sample rigidity associated with current solutions available on the market while decreasing total assay time and preserving throughput. We believe that ExiStation will be able to expand the applicability of molecular diagnostic methods in clinical laboratories and accelerate its adaptation.

ID29. Results of a Pilot Internet-Based, Peer-Group Quality Control Program for HIV-1 Viral Load Testing

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Introduction: Many laboratories performing HIV-1 viral load by nucleic acid testing routinely monitor results of quality control (QC) samples other than those provided by the manufacturer. However, this is done independently without sharing of QC data between laboratories. In 2001, NRL (Melbourne, Australia) developed an Internet-based QC monitoring program specifically designed for molecular and serological infectious disease testing. This program (EDCNet; *http://www.nrlqa.net*) allows laboratories testing the same QC sample in the same test system to compare results and investigate abnormal QC test results in real time. **Methods:** A total of 15 U.S. laboratories were enrolled in the pilot study comprising of Veterans Affair, private and major public health laboratories that performed HIV-1 viral load testing using the Abbott RealTime HIV-1 RNA PCR (Abbott) and/or Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test version 2.0 assays (Roche). Each laboratory tested the same batch of a low

and medium positive, independent QC sample, OptiQual (Life Technologies, AcroMetrix, Benicia CA). Results of the QC samples, along with reagent lot numbers, date of testing, operator and instrument identification were entered into EDCNet. **Results:** A total of 137 QC test results for the two levels of OptiQual were submitted to EDCNet; 87 from the Abbott assay and 50 from the Roche assay. The mean log10 viral load result for the OptiQual low positive QC was 2.56 (Abbott; n=44) and 2.68 (Roche; n=26). The mean QC result for the OptiQuant medium positive QC was 3.88 (Abbott; n=43) and 4.21 (Roche; n=24). The percentage coefficient of variation for the low and medium OptiQual QC tested on the Abbott and Roche assays was 5.08, 4.64, 4.48 and 2.38% respectively. **Conclusions:** Testing QC samples independent of those provided by the assay manufacturer is recommended by many regulatory authorities worldwide. As testing QC samples is relatively expensive, peer-to-peer comparison of QC data allows laboratories to obtain the greatest possible value from QC testing and to maximize the number of data points available for investigation of unusual results. The collection of additional information such as kit lot number, instrument and operator details further aids investigations. EDCNet is used by more than 700 laboratories in over 30 countries. It underpins national QC programs for infectious disease testing in Australia, Canada, Poland and Malaysia. This pilot study demonstrated the utility of EDCNet to collect, store and analyze OptiQual QC test results from US-based laboratories testing for
HIV-1 viral load.

ID30. Detection of Human Papillomavirus from Cervical Specimens by Five Different Assays

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Introduction: Repeated cervical infection with Human papillomavirus (HPV) is a well-known risk factor for cervical cancer, thus early detection of cervical HPV infection is an important mean to prevent cervical cancer. We evaluated five assays utilizing different principles for the detection of cervical HPV infection. **Methods:** A total of 230 cervical swab specimens were collected from subjects with cytologic results of normal (group 1, n=78), ASCUS (group 2, n=51), LSIL (group 3, n=46), and HSIL (group 4, n=55). DNA was extracted from all samples and tested for following assays: two real-time PCR assays (RealTime High Risk HPV by Abbott, reporting 16, 18 and other 12 high risk genotypes; AdvanSure HPV by LG Life Science, detecting 16, 18 and other 39 high and low risk genotypes), hybrid-capture assay (Digene, detecting 8 high risk genotypes), liquid beads microarray (GeneFinder HPV by Innomeditech, reporting 43 detailed high and low risk genotypes), and DNA microarray (PANArray HPV by Panagene, reporting 32 detailed high and low risk genotypes). **Results:** Among the 230 specimens, 15 (6.5%) were negative and 70 (30.4%) were positive for all the five assays. Remaining 145 (63.0%) showed different results according to the assays. Genotype 16 and 18 were detected in around 13% and 5% regardless of assays performed. Positive rate was 44.3% for the Hybrid Capture (high risk genotypes: 10.3%, 25.5%, 65.2% and 92.7% in group 1 to 4), 42.2% for the RealTime High Risk HPV (high risk genotypes: 14.1%, 27.5%, 50.0% and 89.1% in group 1 to 4), 85.7% for the AdvanSure (high and low risk genotypes: 88.5%, 70.6%, 93.5% and 89.1% in group 1 to 4), 75.2% for the GeneFinder (high risk genotypes other than 16/18: 24.4%, 19.6%, 50.0% and 56.4%; low risk genotypes: 28.2%, 9.8%, 26.1% and 18.2% in group 1 to 4), and 49.1% for the PANArray (high risk genotypes other than 16/18: 3.8%, 15.7%, 41.3% and 34.5%; low risk genotypes: 3.8%, 9.8%, 13.0% and 7.3% in group 1 to 4). Genefinder detected 62 (79.5%) cases coinfected with different HPV genotypes, while PANArray and RealTime High Risk HPV assays respectively detected 19 (24.4%) and 11 (14.1%) coinfection. **Conclusions:** Recently developed assays for the detection of HPV showed high positive rates. All the five assays showed similar positive rates in detecting genotype 16 and 18, although overall concordance between the results for other HPV genotypes was not high. These assays may be sufficiently used for HPV testing.

ID31. Verification of the COBAS® AmpliPrep/COBAS® TaqMan HIV Test ver. 2.0

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Introduction: Verification of quantitative test systems in the clinical laboratory is essential in providing quality patient care. Recently, Roche obtained approval for a COBAS® AmpliPrep/COBAS® TaqMan HIV Test Version 2.0. Here we present data for verification of this new version of the assays as it claims a linear dynamic range of 20-10,000,000 copies/ml and additional detection of HIV-1 Group O species. **Methods:** Verification studies were performed in two independent laboratories following the Roche Assay Verification Activities Plan. The Optiquant HIV-1 RNA quantitative panel (Acrometrix, Benicia, CA) was used to assess accuracy, linearity, precision and limit of detection. This panel consists of six samples spanning the range of 0-5x10^6 HIV-1 copies/ml. Accuracy and precision was assessed using a high and low control run in triplicate over a 5 day span. Linearity was assessed by testing the Optiquant panel in triplicate within a single run. Limit of detection was assessed using a panel member at the lower limit of detection for the assay (20 cp/ml) within a single run in 20 replicates. In addition, 40 de-identified, frozen clinical samples were tested to assess method correlation. Results were compared to previously obtained results using the COBAS® AmpliPrep/COBAS® TaqMan HIV Test ver. 1.0. **Results:** The interlaboratory results were nearly identical. Precision was assessed at 2% and 1% Total (Log 10) %CV for low and high controls, respectively. Accuracy measured at two levels over 5 days yielded a mean Log 10 difference (expected to observed) at -0.13. Linearity studies resulted in a regression of y=0.9928x+0.023, with a correlation coefficient (r2) of 0.9989. The limit of detection was demonstrated to be accurate within 95% confidence. **Conclusions:** Analysis of data obtained with the COBAS Ampliprep/COBAS Taqman HIV-1 Test ver. 2.0 verified manufacturers' claims stated in the package insert. Furthermore, correlation of results ensures accurate assessment of HIV-1 viral load in patients can continue without the need for re-baselining. The decreased lower limit of detection and detection of Group O will enable laboratories to better assist in the care of HIV infected patients. The verification process, although timely, provides a precise mechanism to establish the parameters necessary to deliver high quality of care.

ID32. Classification of *E. coli* Based on the Pathogen Genes in Amniotic Fluid

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Introduction: Bacterial contamination of amniotic fluid (AF) can lead to a preterm delivery or rupture of membrane. Some of the *Escherichia coli* strains have toxin and can work as a pathogen in AF. *E. coli* consists of four major phylogenetic groups designated as A, B1, B2, and D. Usually, the commensal strains are placed into the phylogenetic groups A and the extraintestinal pathogenic strains into group B1, B2 and, to a lesser extent, group D. Amniotic fluid infection is usually detected by bacterial culture or bacterial 16S rDNA PCR assay. Because of PCR regents such as Taq polymerase containing *E. coli* DNA, it is difficult to detect *E. coli* from amniotic fluid in 16s rDNA PCR assay. In this study, we have developed a detection method to find pathogen *E. coli* in the amniotic fluid **Methods:** TotalPlex PCR-amplified biotinylated *E. coli* genes (*chuA, TspE4.C2, yjaA*) were hybridized to bead. Based on the present or absent of the *E. coli* toxin gene, *E. coli* were classification into group A, B1, B2 and D. **Results:** Among the tested 30 AF samples, the classification results are as follow; 6 for group A, 5 for group B1, 12 for group B2 and 1 for group D. **Conclusions:** TotalPlex amplification and bead array system can precisely figure out the present or absent of the *E. coli* toxin gene.

ID33. A Bead-Based Multiplex Assay for the Detection of Pathogens Causing Bloodstream Infections

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Introduction: Bloodstream infections (BSI) are severe infections caused by a variety of bacterial and fungal species. BSI are usually diagnosed through the use of blood cultures (BC), a process that can take up to two days (or longer for fastidious organisms) to specifically identify the causative agent. Recently, DNA-based approaches have offered an alternative to traditional BC and provide results in significantly less time (i.e. hours as opposed to days). In this study, a bead-based multiplex assay (the xTAG® Bloodstream Pathogen Panel or BPP) under development by Luminex Molecular Diagnostics (LMD) was evaluated. This multiplex panel is designed to target 20 of the most common pathogens causing BSI (7 Gram-positive and 8 Gram-negative bacterial targets and 5 Candida species), 3 common resistance markers (*mecA, vanA* and *vanB*) and 1 internal/run control and provide an answer within 3 hours. **Methods:** The xTAG BPP was evaluated for both specificity and sensitivity using purified pathogen isolate DNA obtained from the American Type Culture Collection (ATCC). Additionally, preliminary limit of detection (LoD) studies using diluted DNA specimens were performed. Finally, DNA extracted from de-identified positive blood cultures provided by University of Pittsburgh were analyzed and the results compared to traditional typing methods. **Results:** Thirty-five DNA isolates were tested using the BPP, 23 representing the specific targets of the assay and 12 potential cross-reacting species. All positive samples tested were correctly identified (23/23, 100% sensitivity) and there were no false positive results observed within the multiplex for specific targets, nor in any of the potential cross-reacting isolates (35/35, 100% specificity). Preliminary LoD performed using *Staphylococcus aureus*-MRSA strain FPR3757 (ATCC BAA-1556D-5) suggests that for this target in a purified system, single-genome copy (i.e. <10 fg) detection is possible. Twelve blood cultures (including one negative growth control) were also evaluated using the BPP; all 12 were in agreement with the phenotypic results provided by the site. Additionally, the multiplex

panel detected possible unreported co-infections in two of the twelve samples. **Conclusions:** This study showed that the xTAG BPP is highly specific and sensitive for the detection of bacteria and fungi known to cause bloodstream infections. This warrants future evaluation of the xTAG BPP for its utility in patient management.

ID34. Evaluation of 16S rDNA Sequencing Analysis for the Identification of *Vibrio vulnificus* Species

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Introduction: To evaluate the usefulness of 16S rDNA sequencing analysis for the differentiation of *V. vulnificus* and *V. navarrensis*, we tested 57 Vibrio strains by several molecular techniques. **Methods:** Fifty-two clinical and environmental strains and 5 Vibrio reference strains were used in this study. We performed 16S rDNA sequencing analysis, *gyrB V. vulnificus*-specific PCR (gyrB Vvu PCR), *gyrB V. navarrensis*-specific PCR, (gyrB Vna PCR), *V. vulnificus*-specific PCR (vvh PCR), and DNA J sequencing analysis. We determined final consensus identification of 57 Vibrio strains by comparing the results of each tests. We evaluated the usefulness of 16S rDNA sequencing analysis based on the final identification. Three methods of bacterial allocation criteria for species identification by 16s rDNA sequences are as follows: The bacterial allocation method 1 is to allocate the bacterial species showing highest % similarity with tested sequence as its identification. The method 2 is based on the mimicking criteria of CLSI guideline for interpretive criteria for identification of bacteria by DNA target sequencing (99.0% identity for species identification with greater than 0.8% separation between different species). The method 3 is based on the phylogenetic tree analysis. **Results:** The composition of final identification of 57 *Vibrio* strains were 50 (78.1%) *V. vulnificus*, 3 (4.7%) *V. navarrensis*, 2 (3.1%) *V. parahaemolyticus*, 1 (1.6%) *V. cholerae*, 1 (1.6%) *V. mimicus*. By bacterial allocation method 1, 9(18%) and 39(78%) of 50 *V. vulnificus* were identified as *V. vulnificus* and *V. navarrensis*, respectively. One (33.3%) of 3 *V. navarrensis* was identified as *V. navarrensis*. Only 9 strains of *Vibrio* were identified by method 2, of which six were *V. vulnificus*. Fourty-eight of 57 strains could not meet the criteria of method 2. By method 3, 33(66%) of 50 *V. vulnificus* strains, each one of *V. cholerae* and *V. mimicus* was correctly identified. **Conclusions:** The result of 16S rDNA sequencing analysis was not satisfactory for the species identification of *Vibrio*. The method 3 was the best method among three methods of bacterial allocation criteria.

ID35. Molecular Assay to Determine the Cervicovaginal Microflora Composition in Healthy Women and Individuals with Bacterial Vaginosis

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Introduction: Bacterial vaginosis (BV) is an imbalance in vaginal microflora characterized by a depletion of normal lactobacilli and overgrowth of various facultative anaerobic bacteria. Clinical symptoms include an increase in vaginal pH, vaginal discharge and unpleasant fishy odor. BV is associated with increased risk of sexually transmitted infections and serious pregnancy complications. BV is very common in women of reproductive age and is one of the most common reasons that women seek treatment from health care providers. The etiology of this condition remains largely unknown. Laboratory diagnosis of BV is mainly based on Gram staining of vaginal smears, microscopic evaluation and manual counts of bacterial morphotypes. This approach is time consuming, subjective, and substantially outdated. We have developed a molecular assay for identification of the major vaginal bacterial species presented in both healthy and disturbed vaginal microflora, which can serve as a reliable test for BV. **Methods:** DNA is extracted from a cervicovaginal sample and subjected to a panel of PCR assays targeting four *Lactobacillus* species: *L. crispatus*, *L. gasseri, L. jensenii*, and *L. iners*, and five BV-associated pathogens: *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera* type1 and type 2, and BVAB2. PCR data are combined and computed together generating an accurate relative evaluation of bacterial species composition in a given clinical sample. **Results:** We have processed 189 cervicovaginal samples collected from BV carriers as well as healthy individuals. BV conditions were diagnosed by participating OB/GYN providers according to established clinical criteria. We have found substantial correlation between our new molecular PCR test results and the health status of sample donors. The vaginal microenvironment of BV patients was generally dominated by mixed populations of *G. vaginalis, A. vaginae, Megasphaera* spp., and/or BVAB2, whereas the vaginal milieu of healthy women was preoccupied by one or less frequently two *Lactobacillus* species in complete agreement with the literature data. **Conclusions:** We have developed an assay for relative assessment of major bacterial species present in normal and abnormal vaginal microflora. This molecular test may offer a significant advantage for clinical BV management.

ID36. Genotype Specific HPV Detection from Formalin-Fixed, Paraffin-Embedded Tissue Samples in an Academic Institution

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Introduction: Identification of HPV using molecular techniques is employed to assess risk for gynecologic malignancy, characterize cutaneous lesions, localize primary sites from metastatic lesions, and enhance prognostic assessment of squamous cell carcinomas of the head and neck. This task is accomplished by detecting the presence or absence of the virus and categorizing the positive results based on high or low risk genotypes. The purpose of this study is to determine the HPV positivity rate and genotype distribution within formalin-fixed, paraffin-embedded tissues tested for routine clinical care. **Methods:** DNA was extracted and PCR amplified using consensus primers directed at the L1 region of the HPV genome. Positive samples were then subjected to restriction enzyme digestion for genotype discrimination. HPV results were correlated with tissue site and pathologic diagnosis if available. Data were retrospectively collected for all routinely tested specimens over an 18 month period. **Results:** Overall, 92 samples (52 head and neck cases, 35 anogenital biopsies, and 5 other samples) were tested. 55 cases (59.8%) were positive for HPV: 32 (58.2%) high risk, 15 (27.3%) low risk, and 8 unknown risk. 10 separate HPV subtypes were identified. 6 samples contained a coinfection in which two distinct subtypes could be identified. Type 16 was the most prevalent high risk genotype detected and type 6 was the most prevalent low risk type. Of interest, rare genotypes detected included 45, 57, 58, 62, and 66. 55.8% of head and neck cases were HPV positive with 8 genotypes identified. The most prevalent genotype was 16 (58.6%) followed by types 33 (10.3%) and 6 (10.3%). Type 16 was identified in 78.9% of HPV positive samples in this category with a pathologic diagnosis of *in situ* or invasive squamous cell carcinoma. Of the anogenital biopsies, 64.7% were HPV positive with 5 genotypes identified. 50% of positive samples contained type 6 and 22.7% contained type 16. **Conclusions:** HPV testing was predominantly requested on two groups of tissue samples: head and neck specimens and anogenital biopsies. HPV positivity rate in head and neck specimens was 56% with high risk types 33, 45, 58, 62, and 66 being detected in addition to type 16. HPV positivity rate in anogenital samples was 65% with types 57, 58, 61, and 66 being detected in addition to type 16. The only low risk types detected in all specimens were 6 and 11. No positive cases were identified as type 18 or 52.

ID37. Evaluation of FFPE Tissue Specimens with a 24-Plex Luminex Fungal Panel Using the MagPix Instrument

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Introduction: Fungal infections are often associated with suppurative or non-suppurative necrosis, abscess, granulomatous or erosive inflammation. Histopathological diagnosis can be limited by the degree or type of inflammatory infiltrate, the number of fungal organisms and problems with morphological identification. Molecular based detection in formalin-fixed, paraffin-embedded (FFPE) samples can be nonspecific secondary to the reagent contamination or contaminant and commensal fungal spores. We investigated the Luminex fungal ASR panel on formalin-fixed tissue specimens (FFPE) with known fungal infections as also tissue specimens with necrosis and no documented fungal infections to validate and determine the specificity of the ASR panel. **Methods:** 40 positive and 13 negative formalin-fixed, paraffin-embedded tissue sections were selected and fungal elements or necrotic areas were isolated using the Arcturus Laser Capture Microscope and were incubated in the presence of proteinase K and lyticase enzyme. Additionally, evaluation was also performed using whole tissue curls (10 sections of 5 µm thickness) and extracted with a modified protocol using the Qiagen DNA Mini kit. The samples were amplified with Luminex ASRs in a Multiplex PCR format with a 24-plex primer mix to detect *Candida* species *C. albicans, C. glabrata, C. lusitaniae, C. tropicalis, C. parapsilosis, C. guilliermondii* and *C. krusei*, *Aspergillus* species *A. terreus, A. fumigatus, A. flavus* and *A. niger*, *H. capsulatum, C. immitis, C. neoformans, B. dermatitidis, S. apiospermum, S. prolificans, Fusarium, R. microsporus, R. arrhizus, M. indicus, C. bertholletiae,* and *P. Jirovecii* on the MagPix system after nucleic acid extraction with the plate heated to 45°C; 100 beads/set were collected. Results were correlated with histology and culture results. **Results:** Tissue curls were the specimen of choice and sensitivity was improved with the size and thickness of the specimen submitted. 12 of 13 necrotic specimens with no fungal elements identified by culture or histology were negative. One specimen was positive for *C. albicans*. Three specimens were positive for organisms not identified by histology (*Candida* and *Cunninghamella spp* identified instead of *Histoplasma* and *Aspergillus*). Organisms correctly identified included *Aspergillus, Cryptococcus, Histoplasma, Pneumocystis, Candida* and *Coccidioides* with a specificity of 98%. Co-infections included *A. fumigatus* with

C. albicans. **Conclusions:** The Luminex ASR panel had good specificity and sensitivity. The rapid detection in a multiplex format is very helpful in the fast and accurate diagnosis of fungal infections in histological specimens.

ID38. Detection of *Bordetella pertussis* and *Bordetella parapertussis* by a Real-Time PCR in a Community Hospital

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Introduction: Whooping cough or *B. pertussis* continues to be an important cause of morbidity in this country despite the availability of a vaccine. The goal of this evaluation was to confirm the use of a 2-target real-time PCR assay that would detect *B. pertussis* and *B. parapertsusis* from patient samples. **Methods:** A total of 625 patient samples submitted for *B. pertussis* were tested. DNA extraction was performed using the Qiagen EZ1 Advanced XL. Following extraction, 5µl of the sample was tested for the IS481, plS1010, and the ptxS1 targets for *B. pertussis* and *B. parapertussis*. Real-time PCR was performed using the AB 7500 fast DX instrument. **Results:** Of the 625 samples tested, 28 were positive for *B. pertussis*, 7 were positive for *B. parapertsussis* and 24 samples were considered indeterminate. Of the indeterminate samples 1 tested positive for *B. holmesii* by PCR and one sample was considered indeterminate for *B. parapertussis*. Of the remaining 22 indeterminate samples, 13 were siblings of patients that tested positive for *B. pertussis*. **Conclusions:** Real-time PCR is an invaluable tool for the rapid diagnosis of *B. pertussis* and *B. parapertussis* and can affect patient management. Additionally, this 2-target real-time PCR assay can enhance the epidemiological surveillance of *B. pertussis*.

ID39. The Identification of Clinically-Important Yeasts in Blood Cultures Using Pyrosequencing

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Introduction: Our laboratory has developed a method of rapid, selective sequencing of a variable region of the 28S rRNA gene for the identification of clinically-important yeast species using PCR followed by pyrosequencing. In this study, we expanded our current in-house database that includes *C. albicans* , *C. glabrata*, *C. parapsilosis* complex, *C. tropicalis*, *C. lusitaniae*, *C. krusei*, *C. dubliniensis*, *C. guilliermondii* complex, *Cryptococcus neoformans*, and *Cryptococcus laurentii* complex and evaluated pyrosequencing and our updated database to perform direct yeast identification from blood cultures. **Methods:** Twenty-three well-characterized yeast isolates representing *C. kefyr, Rhodotorula sp.* and *Saccharomyces cerevisiae* were tested for addition to our database. Forty-four positive blood cultures from a frozen collection were tested as unknowns. Extracts for isolates and blood culture broths (BactALERT® 3D Fan bottles) were processed using glass beads in lysis buffer, heating at 100°C, then extraction with the MagNA Pure LC or EasyMag System. Real-time PCR was performed on the Rotor-gene 6000 targeting a variable region of the *28S* rRNA gene. Pyrosequencing was performed according to the manufacturer's instructions, using a conserved sequencing primer that was adjacent to a variable region. Pyrograms were evaluated by the instrument software and sequences were manually verified if questionable. Forty bases were used for isolate categorization and results were compared to our updated database. **Results:** Seven *C. keyfr*, 6 *Rhodotorula sp.* and 10 *S. cervisiae*, when blasted against GenBank, were correctly assigned to their respective molecular group, however, for *Rhodotorula sp.* and *S. cervisiae*, identification was possible only to the genus level. For 44 blood cultures, 39/44 (88.6%) had a correct match in our database including one specimen that required repeat testing. These 39 included 12 *C. albicans,* 13 *C. glabrata*, 6 *C. parapsilosis* complex, 3 *C. tropicalis*, 2 *Cryptococcus neoformans*, 1 *Rhodotorula sp.* and 2 *Saccharomyces sp.* Of the 5 remaining samples that did not match our database, 4 blood cultures containing *C. albicans* had a 39/40 (97.5%) match in GenBank for *C. albicans.* One blood culture containing *C. lusitaniae* and coagulase negative *Staph sp.* resulted in a pyrosequence that did not have a match in GenBank or our database with only 29 readable bases. The yeast isolate was tested directly and matched the blood culture pyrosequence result. **Conclusions:** Pyrosequencing using our expanded in-house database can be used to identify yeast directly from blood cultures.

ID40. Clinical Evaluation of the BD MAX™ MRSA Assay, the BD GeneOhm™ MRSA ACP Assay and Conventional Culture for the Rapid Detection of MRSA Directly from Nares Swabs

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Introduction: *Staphylococcus aureus* (SA), a major cause of nosocomial infections, is commonly found in the nose or on the skin of healthy individuals (asymptomatic carriers). Methicillin-resistant strains of SA (MRSA) are frequently encountered in health-care settings, and the prevalence continues to increase. Development of sensitive molecular amplification techniques allows for detection of only a few copies of bacterial DNA in clinical specimens in a few hours. The combination of sensitivity and rapidity allows for the prompt isolation of MRSA carriers and thus prevention of the spread of antibiotic resistant SA. This study evaluated two molecular methods (BD MAX™ MRSA Assay and BD GeneOhm™ MRSA ACP Assay) and conventional culture for direct detection of MRSA from nasal swab specimens. Both the BD MAX™ MRSA Assay (BD MAX™) and the BD GeneOhm™ MRSA ACP Assay (ACP) utilize real-time PCR for the amplification of MRSA DNA and fluorogenic target-specific hybridization probes for the detection of amplified DNA. The BD MAX™ is a fully automated sample-to-result platform that is currently investigational use only. The ACP assay is performed on the Cepheid SmartCycler® instrument and is commercially available. **Methods:** A total of 96 paired-nares specimens were collected in Liquid Stuart media. One swab was tested using the ACP assay according to package insert instructions. The second swab was inoculated on to a CSA and blood agar media and then broken into a sample buffer tube, vortexed, and analyzed using the BD MAX™ instrument/assay. Additionally, an aliquot of the sample buffer was inoculated into an enrichment broth (TSB with 6.5% NaCl). Direct and broth-enriched cultures were identified using standard and chromogenic media, latex agglutination, and cefoxitin disk diffusion methods for MRSA. Specimens were analyzed within 5 days of collection. Results from ACP, BD MAX™ and culture were used to calculate sensitivity/specificity using patient infectivity status (PIS) with true positives defined as any two out of three positive results. **Results:** Of the 96 subjects tested to date, 29 yielded true positive results and 62 yielded true negative results based on PIS. Both ACP and BD MAX™ exhibited 100% sensitivity and 98% specificity respectively, while culture yielded 83% sensitivity and 97% specificity based on PIS. **Conclusions:** Both BD MAX™ and ACP exhibited excellent sensitivity/specificity for direct detection of MRSA from nasal swab specimens. The fully-automated BD MAX™ accommodates up to 24 specimens and returns results in ~3h allowing rapid decisions affecting patient management.

ID41. Establishment of an *IL28B* Genotyping Assay for Clinical Use

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Introduction: Chronic hepatitis C (CHC) infection currently affects approximately 170 million people worldwide and is the leading cause of cirrhosis in North America as well as an important risk factor for hepatocellular carcinoma. Standard of care treatment for HCV infection, pegylated interferon alpha and ribavirin (PegIFN/RBV), fails to achieve sustained viral response (SVR) in >50% of the HCV genotype 1 infected individuals. Recently, numerous studies have identified two allelic variants, rs12979860 and rs8099917, upstream of the interleukin-28B (*IL28B*) gene that are correlated with the rate of SVR following PegIFN/RBV therapy. CHC patients with the favorable CC genotype of rs12979860 have a 2- to 3-fold greater rate of SVR following PegIFN/RBV therapy than individuals with the CT or TT genotype. Similarly, patients with the TT genotype of rs8099917 have higher rates of SVR following combination PegIFN/RBV therapy as compared to GT or GG genotype individuals. We report on our initial validation of a clinical genotyping assay for the rs12979860 and rs8099917 polymorphisms in the *IL28B* locus. **Methods:** Genomic DNA was extracted from 100 de-identified residual EDTA whole blood samples with the EZ1 DNA Blood kit on the Qiagen EZ1 BioRobot. Genomic DNA was analyzed for yield and purity by absorbance spectroscopy. The extracted DNA was then analyzed using TaqMan® real-time PCR primers and probes on the Applied Biosystems 7500 Fast Real-Time PCR System with probes specific for the C and T alleles of rs12979860 and the G and T alleles of rs8099917 in the *IL28B* locus. **Results:** All 100 samples analyzed generated clinically usable results. In this set of de-identified patient samples 41/100 had the favorable CC genotype, 50/100 were CT and 9/100 were TT at rs12979860: overall allele frequency was 66% C and 34% T. For the rs8099917 polymorphism, 60/100 were the favorable TT genotype, 36/100 were GT and 4/100 were GG: overall allele frequency was 22% G and 78% T. The allelic frequency is similar to that reported in the HapMap project for a Caucasian population (rs12979860 C=74.2% T=25.8%; rs8099917 G=16.7%, T=83.3%). **Conclusions:** Our initial validation studies of a laboratory developed *IL28B* genotyping assay using TaqMan primers and probes demonstrates that this assay will be readily applicable to clinical testing, demonstrating allelic frequencies that are comparable with previous reports. Clinical availability of this assay will allow the clinician, in conjunction with other clinical factors, to predict the individual patient's response to PegIFN/RBV therapy.

ID42. Evaluation of the Focus Diagnostics Simplexa™ Flu A/B & RSV Direct Real-Time PCR assay

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Introduction: Influenza A (Flu A), influenza B (Flu B) and respiratory syncytial virus (RSV) are clinically important viral pathogens that cause respiratory illness in millions of individuals worldwide each year. The Simplexa Flu A/B & RSV Direct assay is in development for the differential detection of Flu A, Flu B and RSV from nasopharyngeal swabs (NPS) without extraction. The goal of this study was to compare the performance of the Simplexa Flu A/B & RSV Direct assay to culture, and to a real-time PCR methodology that uses automated nucleic acid extraction. **Methods:** Performance of the Simplexa Flu A/B & RSV Direct assay was assessed with panels of clinical NPS specimens, and the results were compared to previous culture and/or qualitative real-time PCR results. Specificity studies were performed using a contrived panel of 31 respiratory pathogens and normal flora. Limit of detection (LoD) studies were performed using serially diluted viral stocks, and the LoD was determined as a concentration where ≥95% replicates were detected. In addition, assay inhibition/interference was determined by spiking relevant concentrations of potentially interfering substances. **Results:** The Simplexa Flu A/B & RSV Direct assay showed 100% agreement for all clinical specimens that were reported positive by both culture and the comparator real-time PCR assay. Negative agreement for Flu A, Flu B and RSV were 98.4% (186/189), 99.5% (187/188) and 100% (202/202) respectively. The multiplexed assay was specific and no cross-reactivity was observed with other organisms. The assay detected geographically diverse strains of Flu A including the H1 and H3 subtypes and the 2009 pandemic H1N1 strain. It was also capable of detecting all tested strains of Flu B, RSV-A and RSV-B, and the analytical sensitivity for each of the three targets was less than 30 TCID50/mL. Studies using clinically relevant interferents did not show any inhibition of target detection. **Conclusions:** The sample-to-answer Simplexa Flu A/B & RSV Direct can provide results from 8 samples in approximately 1 hour with minimal hands-on manipulation. The assay displays similar performance to conventional detection methods, including a real-time PCR method that required nucleic acid extraction.

ID43. [WITHDRAWN]

ID44. Lack of a BK Virus DNA International Standard Complicates Comparison of Results Between Laboratories and Development of a Lab-Developed Quantitative Assay

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Introduction: BK virus (BKV)-induced nephropathy causes allograft loss in approximately 5% of renal transplant patients. Risk of allograft loss increases with high viral load (VL); monitoring plasma VL is used to detect viremia and allow for immunosuppression reduction to prevent allograft loss. Cut-off values for use in this monitoring paradigm have been recommended but have been difficult to implement due to quantification variability between different assays in the absence of an international standard that can be used as a uniform calibrator. Assay-dependent quantitative variability is particularly problematic for laboratories transitioning from reference laboratory testing after developing their own assays and for patients monitored at multiple laboratories. **Methods:** Split plasma samples (n= 136) were tested by the Qiagen artus BK Virus RG PCR Kit (artus) and at least one other method including Eragen Multi-code BK Primer ASR (Eragen), a reference laboratory (RefLab1) using Nanogen ASR reagents, reference lab 2 (RefLab2) using Focus ASR reagents, or in-house testing using Focus ASR reagents. Calibrators included artus QS standards, Zeptometrix BK Virus Linearity Panel, Acrometrix Quantification Panel, serially diluted Applied Biotechnologies BK Viral DNA (ABI), and linearized plasmids (RefLab1 and RefLab2 only). Selected samples were genotyped by direct sequencing. **Results:** BK VL measured by artus were about 1-log lower than RefLab1, with excellent linear correlation; similar bias was observed with commercial panels. Plasmas (N=40) were tested by 4 methods: artus using 4 different standard curves, Eragen with Zeptometrix standard curve, RefLab1, and RefLab2. No bias was observed between artus and Eragen results or between RefLab1 and RefLab2. However, a 0.5-2.2 log bias was observed between artus and both reference labs. The artus results quantified using different standard curves were similar, except with ABI calibrators that showed a bias of 0.5-log. A subset of samples was genotyped by sequencing including those with the largest and smallest observed bias. Samples with genotypes 1a, 1b1 and 1b2 showed a 1-log bias between artus and RefLab1; while no quantification bias was observed for genotype 4 suggesting that RefLab1 method under-quantifies genotype 4. **Conclusions:** Comparison of several BKV DNA quantification methods demonstrated quantitative bias, but overall good correlation. Quantitative bias was likely due to the use of different calibrators for each assay although some assay designs may under-quantify genotype 4. The observed bias highlights the need for BKV international standards. Inability to standardize quantification complicates validation of laboratory developed tests and clinical management of patients monitored in multiple labs.

ID45. Mupirocin Resistance in Methicillin-Resistant *Staphylococcus aureus* (MRSA) Colonized Residents in Long-Term Care Facilities (LTCFs)

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Introduction: One approach to MRSA control is surveillance for asymptomatic carriers and then decolonization of those found MRSA positive using intranasal mupirocin. The goal of this study is determine the prevalence of mupirocin resistance in LTCFs. **Methods:** This study is part of a cluster randomized trial to control MRSA disease in 12 nursing units at 3 different LTCFs. A pre-moistened double swab of both nares was obtained from all residents for a point prevalence survey. Real-time PCR (qPCR) was performed using one of the nasal swabs and if the PCR test was positive for MRSA, the second swab was cultured onto agar and the recovered MRSA tested using another qPCR test for mupirocin resistance and the presence of PVL (as a marker for community-associated MRSA). A commercial qPCR system was used to detect MRSA and an in-house qPCR assay was for identifying mupirocin resistance and the presence of PVL. **Results:** Overall, 680 residents were tested and 111 MRSA positive (prevalence = 16.5%). Mupirocin resistance was detected in 16 (14.4%) and PVL in 4 (3.6%). Importantly, the two LTCFs serving an acute care system that actively decolonizes MRSA carriers as part of a comprehensive surveillance program had mupirocin resistance rates of 20% and 39% in their MRSA colonized residents. The LTCF not serving this same acute care organization had 2% mupirocin resistance ($p = 0.008$ and 0.001 comparing the two LTCFs with high resistance to the one with 2% resistance, respectively) **Conclusions:** qPCR can rapidly detect MRSA as well as mupirocin resistance and PVL in the residents of LTCFs. Mupirocin resistance may be significantly increased for LTCF residents coming from acute care facilities with an active MRSA decolonization program. Additional therapeutic agents are needed for decolonization if mupirocin continues to be used in MRSA control programs. The prevalence of community-associated MRSA remains low in many LTCFs, suggesting most of their strains arise from the healthcare system and not the community.

ID46. Comparison of Hybridization Probe-Free and Hybridization Probe-Based Assays for the Quantitative Detection of Cytomegalovirus

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Introduction: Cytomegalovirus is a member of the *Betaherpesviridae* family capable of establishing lifelong persistent and latent infection after the primary exposure. CMV can reactivate leading to asymptomatic viral shedding or disease development. In immunocompetent individuals the infection is held under control by the host's immune system, CMV infections are rarely serious and cause only mild symptoms resembling those of mononucleosis lasting up to a few weeks. Serious CMV disease is generally restricted to immunologically immature or immunocompromised hosts. CMV is a major cause of lung, liver, gastrointestinal renal and retinal disease in solid organ transplant patients and hematopoietic stem cell transplant patients. Monitoring CMV load is important to assess response to therapy, to identify patients who are likely to have recurrent disease and to optimize pre-emptive treatment. During the last decade real-time PCR based quantitative DNA assays has became the method of choice for CMV viral load monitoring. These assays employ a wide variety of PCR chemistries and differ in the modes of the detection of amplified products. **Methods:** We compared the parameters of a probe–free real-time PCR assay (MultiCode RTx CMV Primers, EraGen Biosciences) with those of a probe based assay (CMV UL54 Primer/Hybridization Probes, Roche) using OptiQuant CMV calibration panels (AcroMetrix) and de-identified patient serum samples. Viral DNA was extracted using a QiaCube automated nucleic acid extractor (Qiagen), and real-time PCR reactions were performed and he amplifications detected on the LightCycler 2.0 (Roche) platform. . Statistical analysis and correlation was calculated using MedCalc biostatistical software (MedCalc). **Results:** The linear range for both assays exceeded 5 logs. The low limit of detection of the probe-based assay was 1000 copies/ml (92% confidence n=23), while that of the probe-free assay was 250 copies/ml (100% confidence, n=20). Testing clinical samples, the positive agreement between the two assays was 100% with good correlation between the quantitative results (r=0.9123, n=27), while the negative agreement was 87% (n=15). The results of reproducibility, specificity and cross-reactivity studies were similar for both reagent sets. **Conclusions:** The probe-free CMV assay appears to have higher sensitivity than the probe-based assay. Other assay

parameters including reproducibility, clinical specificity and assay linearity are comparable.

ID47. Impact of Pap + HPV Co-Testing: Data from the First Year

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Introduction: High risk human papilloma virus (HPV) subtypes have been implicated as the causative agent in cervical cancer. As such HPV testing has long been performed on cervical pap samples with atypical cytopathology results (ASCUS), concerning for risk of cancer. Recently, pap + HPV co-testing has been recommended as a screening test for individuals age 30 and up for early identification of risk. Furthermore, a double negative result carries a very high negative predictive value. Due to the increased sensitivity of pap + HPV co-testing over pap screening alone, a lengthened testing interval of 3 years is recommended. We proposed a three-year interval for pap + HPV co-testing for patients age ≥30 and pap alone for patients age 21-29. We predicted that this testing guideline would improve care by providing a test with increased sensitivity to the population at the highest risk for cervical cancer and decreasing the incidence of "over-papulation" in younger patients, which by extension should reduce over treatment of this group. With the lengthened screening interval we also expected a decrease in our pap volume. **Methods:** Clinicians collecting pap smear samples were educated about the implementation of the new pap + HPV co-testing guideline for individuals age ≥30. Samples for pap analysis and HPV testing were co-collected for the appropriate population. Pap analysis was continued as previously performed. High risk HPV testing was performed using the Cervista™ HR HPV method. **Results:** During the first 10 months after implementation of co-testing, over 33,000 HPV test have been performed. The overall incidence of high risk HPV was 5.71%. For individuals with normal cytology, the HPV incidence was 3.55% and for individuals age ≥30 years with normal cytology, 3.45%. Pap testing data showed a 13.5% decrease in testing since the implementation of the new screening guidelines. **Conclusions:** The incidence of high risk HPV in individuals age ≥30 years with normal cytology is comparable to other reported studies. We have observed an expected decrease in pap volume with implementation of the new screening guidelines and have also seen a decrease in pap testing for individuals under age 21. Preliminary data suggest that the new screening guidelines are following expected results.

ID48. Multiplex Respiratory Virus PCR: A Comparison of the xTAG® and FilmArray® Respiratory Virus Panel on Brochoalveolar Lavage Specimens

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Introduction: Children, the elderly, and immunocompromised patients are susceptible to respiratory viruses that cause acute local or systemic infections. The clinical presentation may be similar, therefore identification of the causative agent(s) aids in the selection of the appropriate treatment and infection control measures. Several multiplex PCR based assays have been developed for the detection of respiratory viruses. Among these are the FilmArray® (Idaho Technology Inc.) and the xTAG® (Luminex) assays. Performance of these assays was addressed using brochoalveolar lavage (BAL) to detect viruses within each specimen. Both assays detect influenza A (Flu A; seasonal H1 and H3) and influenza B (Flu B), adenovirus, parainfluenza 1-3 (Para 1-3), respiratory syncytial virus (RSV), human metapneumovirus (HMPV) and human rhinovirus/enterovirus (HRV/E). The FilmArray additionally detects coronaviruses (NL63 and HKU1), Para 4, and Flu A subtype 2009 H1. The xTAG also differentiates RSVA and RSVB. **Methods:** 114 BALs were collected from hospitalized patients during winter of 2010-2011. One aliquot was processed with the xTAG and a second previously frozen aliquot was tested by the FilmArray. For the xTAG, viral nucleic acids were extracted using the EasyMag®, reverse transcribed (RT), amplified, and analyzed with Luminex technology. For the FilmArray, BALs were processed in a closed RT-PCR system that isolates, amplifies, and detects the viral targets. **Results:** The xTAG was positive for 24 Flu A (negative for H1/H3), 2 Flu A H3, 2 adenovirus, 2 Para2, 6 Para3, 8 RSVA, 5 RSVB, 39 HRV/E, 7 HMPV and 19 BAL's were negative. The FilmArray was positive for 22 Flu A 2009 H1, 2 Flu A H3, 1 Flu A non-typeable, 2 adenovirus, 2 Para2, 7 Para3, 13 RSV, 35 HRV/E, 7HMPV and negative for 23 BALs. Of the 6 discrepant results, upon repeat testing 3 were positive for HRV/E, and 1 was negative by FilmArray One BAL positive for Flu A by the xTAG was consistently invalid by the FilmArray. One BAL positive for Para 3 by FilmArray was negative by xTAG. Co-infection was found in 5 BALs with the FilmArray (4 with coronavirus and 1 with HRV/E-Para 3). **Conclusions:** Both assays performed comparably with BAL specimens for the majority of the viruses included in both assays. The FilmArray system offered a broader virus detection range and greater ease of use. However, the xTAG allows for greater specimen throughput.

ID49. [WITHDRAWN]

ID50. Improved Performance of Cervical Cancer Screening Using HPV E6, E7 mRNA Quantification with Polygonal Gating and Cellular Normalization

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Introduction: Abnormal cervical cytology has low sensitivity for CIN 2+ disease on biopsy. HPV DNA has been used to help triage abnormal cytology specimens especially ASCUS. HPV DNA, however, is positive in >50% of ASCUS samples yet <10% of ASCUS samples have CIN 2+. Similarly, HPV DNA is positive in >80% of LSIL cases yet <20% have CIN 2+ on biopsy. In this study, we show improved cervical cancer screening test performance by cellular quantification of HPV E6, E7 mRNA simultaneously with measures of cellular size (forward scatter) and complexity (side scatter). **Methods:** Thinprep liquid-based cervical cytology was performed on a total of 115 samples. Residual liquid (1.0 mL) was used to perform E6, E7 mRNA quantification on a cell-by-cell basis using the HPV E6 and E7 mRNA reagents (IncellDx, Inc., Menlo Park, CA). Cell size and complexity were simultaneously assessed using forward and side scatter, respectively. **Results:** Thirty-six ASCUS samples were analyzed and 16.67% showed overexpression of E6, E7 mRNA; 27 LSIL samples were analyzed and 29.6% demonstrated overexpression of E6, E7 mRNA; 5 HSIL samples were analyzed and 40% overexpressed E6, E7 mRNA. Forty-seven NILM samples were analyzed demonstrating a specificity (based on PAP) of 93%. One endocervical adenocarcinoma confirmed by colposcopy and biopsy was detected using E6, E7 mRNA quantification on gated endocervical cells. **Conclusions:** The results presented in this study are in agreement with previous reports (Massad LS, Gynecol Oncol. 2001) demonstrating 9% CIN 2+ in ASCUS cytology, 17% CIN 2+ in LSIL, and 42% CIN 2+ in HSIL cytology specimens. The HPV E6, E7 mRNA reagents offer a robust and user friendly method that can be performed from routine liquid cytology specimens.

ID51. Development of MRSA/MSSA Independent NAT Run Controls for Multiple Test Platforms

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Introduction: Methicillin resistant (MRSA) and Methicillin-sensitive (MSSA) *Staphylococcus aureus* are leading causes of hospital acquired infections. A number of molecular tests have been approved by the FDA to screen hospital patients for MRSA and/or MSSA. An unmet need exists for independent run controls that can be used to monitor test performance and validate test systems using patient-like samples without introducing infectious bacteria into the hospital laboratory. SeraCare has developed ACCURUN® 530 MRSA positive control and ACCURUN® 550 (MRSA and MSSA combination pack) for use in quality control and method evaluation for nucleic acid based surveillance testing. **Methods:** Methicillin resistant (MRSA ATCC# 43300) and methicillin sensitive (MSSA ATCC# 29213) strains were used to formulate these controls. The bacteria are heat inactivated as part of the manufacturing process. These liquid controls are provided with a flocked COPAN® swab. The user dips the provided swab into the liquid, and the swab is then processed in the same manner as a patient sample. The controls have been evaluated with the Cepheid Xpert® SA Nasal Complete assay, the Roche LightCycler® MRSA Advanced Test, and the BD GeneOhm™ MRSA Assay. **Results:** The ACCURUN 530 and 550 controls are formulated to be low positives in order to monitor assay sensitivity; therefore, experiments were performed to determine the lower limits of detection for two popular test platforms. Dilution series of heat inactivated MRSA and MSSA cultures were produced and tested with the Cepheid and Roche assays. The lower limit of detection for the Cepheid assay was estimated to be $5.0x10^2$ colony forming unit equivalents (CFU)/mL (100% positivity for 6 replicates across 3 testing days). The lower limit of detection for the Roche test was similarly estimated to be $1.0x10^3$ CFU/mL. The target concentrations for SeraCare's ACCURUN controls were set at $2.75x10^4$ CFU/mL to allow for detection even with variability in swab use. These controls are designed for storage at 2-8ºC. Testing on the Cepheid platform indicates the products are stable for at least 6 months. Real-time and accelerated stability studies are on-going. **Conclusions:** The ACCURUN 530 and 550 controls provide tools for laboratories to monitor the performance of MRSA surveillance testing and can be used as daily run controls as well as for kit lot acceptance testing, validation activities, and technician competency evaluations.

ID52. Detection of Toxigenic *Clostridium difficile*: Comparison of the Cell Culture Neutralization, Xpert® *C. difficile* and the Illumigene™ *C. difficile* Assays

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Introduction: *Clostridium difficile* (CD) is the most important cause of nosocomial acquired diarrhea. The disease caused by CD ranges from mild, self-limited illness to a

severe, life-threatening colitis. The incidence of CD associated disease has risen dramatically over the last several years. Several laboratory techniques are available to detect CD in fecal specimens. Because questions have been raised about the reliability and turn-around time of the cytotoxin (toxin B) in the cell culture neutralization assay (CCNA), a study was performed comparing the performance of CCNA with two molecular assays. The detection of the toxin B gene (*tcdB*) by real-time PCR (Xpert® *C. difficile*; Cepheid, CA), and the detection of toxin A gene (*tcdA*) by loop-mediated isothermal amplification (Illumigene™, Meridian Bioscience, Inc.) were evaluated. The isolation of *C. difficile* in culture followed by CCNA on the isolate (toxigenic culture -TC) was used as the gold standard for discrepant testing. **Methods:** Two hundred prospectively collected stool specimens were tested simultaneously by the CCNA, Xpert® *C. difficile* and the Illumigene™ *C. difficile* assays. Additionally, 50 retrospective stool specimens (previously positive by CCNA) were included in the study. Discrepant results were tested by TC. **Results:** Of the 200 prospective stools tested, 10.5% (n=23) were positive by CCNA, 17.5% (n=35) by Illumigene™, and 21.5% (n=43) by Xpert®. Of the 50 retrospective stools, previously positive by CCNA, 94% (n=47) were positive by Illumigene™ and 100% (n=50) by Xpert®. Of the 11 discrepant results (negative by Illumigene™ but positive by Xpert®), 10 tested positive by TC and for one specimen, we were unable to isolate *C. difficile* from the stool. **Conclusions:** The Xpert® was the most sensitive, rapid and easy to use assay for the detection of toxigenic *C. difficile* in stool specimens.

ID53. Combined Morphology, Nuclear-to-Cytoplasmic Ratio and E6, E7 mRNA Quantification in Suspension Improves Cervical Cancer Screening Performance

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Introduction: Novel technology presented here can combine cell morphologic analysis as routinely done on slides by PAP smear with biomarker detection while the cells remain in suspension thus maximizing workflow and throughput. **Methods:** The objective of this study was to develop a non-subjective means for cervical cancer screening that includes biomarkers used in separate assays in a single slideless analysis. Using the HPV OncoTect3Dx assay performed on an image cytometer, we used bioinformatics to choose from 600 cellular and molecular parameters that distinguish normal, low grade, and high grade cells and samples from the others. We compared the results from standard liquid-based cytology (Pap smear) with the results from the HPV OncoTect 3Dx assay. **Results:** The combination of nuclear-to-cytoplasmic ratio (N/C ratio) and E6, E7 mRNA defined with 97% accuracy the cytology result on 200 liquid-based cytology samples (NILM, LSIL, and HSIL). Of the six discrepant samples, Four HSIL samples were downgraded to LSIL and two LSIL samples were more likely to be high grade based on marker expression. **Conclusions:** This highly automated analysis that combines and digitizes the criteria used by pathologists to determine cytologic abnormalities in liquid-based cytology specimens with E6, E7 mRNA quantification/cell and DNA ploidy analysis may lead to less subjective means of cervical cancer screening while incorporating the molecular quantification of HPV-encoded oncogenes.

ID54. Performance Evaluation of the Quantitative Real-Time PCR artus® BK Virus RG RUO Kit Using the QIAsymphony SP/AS Module and Rotor Gene® Q Instrument

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Introduction: BK viral load measurement plays a key role in the management of kidney transplant patients. Today, the majority of clinical testing is performed using laboratory-developed tests (LDTs). There is a need for a standardized test to further evaluate the clinical use of BK viral load in clinical practice. We report here the performance characteristics of the artus® BK Virus RG PCR RUO kit for the detection and quantification of BK Virus (BKV) in plasma using the QIASymphony SP/AS module and the Rotor Gene® Q instrument (Qiagen system). Results were compared to our laboratory-developed assay (LDT) for correlation and agreement. **Methods:** Linear range and lower limit of quantification (LOQ) were evaluated using a commercial BKV panel with additional dilutions. In addition, eight replicates each of a pool of anonymized patient specimens with high (6.05 log10 copies/mL) and low (3.49 log10 copies/mL) levels of viral load were tested in 4 different runs for reproducibility. Correlation and agreement studies were performed on 122 anonymized patient specimens previously tested with the LDT. **Results:** The assay was linear from 2.00 to 6.00 log10 copies/mL (based on an SD of 0.20 log10 copies/mL) with an R2 value of 0.9989 (y=1.0801x - 0.4005). High level of reproducibility was obtained for the high and the low pooled patient specimens, with a CV of 1.16% and 2.87% in log10 copies/mL, respectively. A high level of inter-assay precision was also obtained when plasma specimen from 8 patients were repeated in 2 runs performed on different days. Regression analysis showed a high correlation between the methods (R2= 0.9693) for the 92 positive specimens with viral loads ranging from 3.0 to 7.23 log10 copies/mL. A negative bias was obtained when both methods were compared for agreement. On average, the Qiagen reagents yielded BK viral load values that were -0.64 log10 copies/mL lower than the values obtained with the LDT and both methods agreed equally through the range with limits of agreement of -0.16 and -1.11, respectively. Thirty specimens were negative by both methods. **Conclusions:** The Qiagen Quantitative Real-time PCR artus® BK Virus RG RUO kit and QIAsymphony SP/AS module and Rotor Gene® Q instrument provided very good results and was in agreement with our LDT.

ID55. Evaluation of the Walk-Away Multiplex FilmArray Respiratory Panel (RP) Test for Immunocompromised Patients in a Large Transplant Center

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Introduction: Recently, Respiratory Multiplex Viral Panel (RVP) tests have been used to detect more than 12-16 respiratory viral pathogens from a single nasopharyngeal swab (NP swab). These tests accurately detect viral infections in most patients with Influenza Like Illness (ILI) during respiratory season. Unfortunately, the current multiplex assays are labor intense with a long turnaround time for results (usually between 5-9 hrs). They also require expensive nucleic acid extractors and PCR instrumentation with large footprints. We evaluated a newly FDA cleared walk-away FilmArray RP Test (IdahoTechnology) using pre-characterized clinical samples collected from immunocompromised or severely ill patients with ILI symptoms from our large transplant services. **Methods:** Ninety-two samples sent for routine respiratory virus testing (includes PCR and DFA) were tested using the FilmArray RP test. In addition a respiratory viral control panel (NATRVP-3, Zeptometrix Corp) was tested with the FilmArray RP. Approximately 300 μl of sample is inoculated into a lysis buffer. The lysed sample and hydration solution are then inoculated into the FilmArray reagent pouch that contains freeze-dried reagents necessary for sample preparation, RT-PCR, and detection. **Results:** The Film Array RP detected 53% (49/92) of the samples positive for one or more respiratory viruses. Positive results for RP are as follows: 15 respiratory syncytial virus, 13 influenza A, 4 influenza B, 2 adenovirus, 6 parainfluenza, 2 human metapneumovirus, 6 rhinovirus and 9 coronavirus. The RP also detected 9 coinfections, 7 with 2 viruses and 2 with 3 viruses. Our routine methods detected 38% (35/92) with 14 respiratory syncytial virus, 13 influenza A, 3 influenza B, 1 adenovirus, 3 parainfluenza and 2 coinfections with 2 viruses. The RP also correctly identified the 16 respiratory viruses from the respiratory control panel. **Conclusions:** The FilmArray RP performed comparably to our methods on the eight respiratory viruses routinely tested for and in addition it detected a significant number of viruses that are not routinely tested for. The RP is a walk-away system that combines sample extraction , amplification, detection, and results analysis in an easy to use format that requires 2 minutes of hands on time and has a total turnaround time of 1 hour. The FilmArray Test can be easily adopted in a busy hospital laboratory to better manage immunocompromised and very sick patients during the respiratory season.

ID56. Specimen Handling Options for the Detection of *Chlamydia trachomatis* and *Neisseria gonorrhea* Using the Abbott Realtime CT/NG Assay

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Introduction: Nucleic acid amplification for detection of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhea* (NG) is the gold standard for laboratory diagnosis. Automation in molecular assays reduces hands-on time, turn-around-time, potential contamination and facilitates implementation of assays in laboratories with limited technical expertise. We have compared specimen performance of the CT/NG assay on Roche COBAS Amplicor (COBAS) with the automated Abbott Realtime CT/NG (Realtime) assay. **Methods:** A total of 2040 swab and urine specimens were compared in the COBAS and the Realtime CT/NG assays. For COBAS, swabs were collected in M4 transport media and urine specimens were collected in polypropylene containers without preservatives. For comparison with the Realtime, these specimens were assayed in either of the following ways: i) 1696 swab specimens collected in M4 transport media were vortexed and poured into empty tubes without any stabilizing buffer; ii) 89 swab specimens were vortexed and 500ul was pipetted into the Abbott multi-Collect Specimen tube; iii) 200 swab specimens were directly collected using both the Abbott Specimen collection kit and the M4 transport media. 55 urine specimens were collected in clean, empty polypropylene tubes and poured into the multi-Collect tubes or directly in the multi-Collect tubes. **Results:** Of the 120 (7.1%) M4 swab specimens i) that were positive for CT in COBAS, 111/ 1696 (6.5%) repeated as positive in the Realtime assay. Similarly NG was detected in 2.0% of swabs tested in COBAS versus 1.53% in the Realtime assay. In studies in which selected M4 specimens were added directly to the multi-Collect collection tube with stabilizing buffer, ii) 26% and 10.1% that were positive for CT and NG in the COBAS resulted in 27% and 7.8%, respectively, in the Realtime.

When a direct comparison iii) of two different swabs was performed, 6.5% of the specimens were positive for CT in COBAS and 5.9% were positive in Realtime. In comparison 4.8% and 3.7% of the specimens were positive for NG in COBAS and Realtime, respectively. Analysis of 55 urine specimens yielded an increased number of positives: 2.1% for CT and 3.6% for NG in COBAS compared to the Realtime assay. **Conclusions:** The performance of both assays was comparable. Specimens collected in M4 viral transport media can be used directly or added to the specimen collection tube and tested on the Abbott Realtime CT/NG assay. Use of the Realtime automated assay results in a significant time savings in a laboratory.

ID57. Development of a Rapid and Simple to Perform Isothermal Molecular Assay for the Detection of Group B Streptococcus from Lim Broth and Direct Specimens on a Noninstrumented Platform

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Introduction: *Streptococcus agalactiae* (GBS) is the leading cause of neonatal sepsis, pneumonia and meningitis and is present in as many as 35% of pregnant women, most of whom are asymptomatic. To reduce transmission, screening is performed around 35-37 weeks in pregnancy; however, many women become re-colonized during the period of time between initial screening and delivery. Traditional methods for screening include culture, a less sensitive option, and molecular assays, which often are costly and/or difficult to implement. Herein, we describe the development of a sensitive and specific molecular assay for the detection of GBS from Lim Broth and direct specimens using a novel isothermal instrument-free platform. **Methods:** Helicase-dependent amplification (HDA), an isothermal nucleic acid amplification platform that utilizes a helicase to separate strands of DNA or RNA, allows for target-specific amplification in the absence of thermocycling. The GBS assay combines HDA with a disposable self-contained detection device that allows for the rapid detection of amplicons generated by primers and probes specific for a region of the thiolase (*atoB*) gene. The assay is performed from Lim Broth culture as well as from direct vaginal/anogenital swabs. For the Lim Broth assay, an aliquot of the overnight culture is placed into a dilution tube, briefly heated to lyse the cells, and then transferred to a reaction tube containing lyophilized reagents. The sample is incubated at 64°C for 40 minutes and transferred to a cassette wherein the result is read via a lateral flow strip. For the direct assay, a patient swab is diluted and centrifuged. The supernatant is removed, lysed at 95°C and follows the above workflow for incubation and detection. Either assay is performed in less than 1.5 hrs and "on-demand" eliminating the need to batch samples. **Results:** Analytical sensitivity testing has determined that the assay can detect as few as 10 copies of genomic DNA, does not cross-react with any non-GBS organisms that have been evaluated (31/31), and is capable of detecting each of the GBS serotypes evaluated (8/8). A preliminary clinical study performed with previously characterized frozen Lim Broth specimens has found that the assay identified 11/12 positive samples and 37/37 negative samples. The one discrepant sample was confirmed as a true negative by alternate PCR. **Conclusions:** The HDA-based assay for the qualitative detection of GBS is a sensitive and specific assay that can be run in a wide variety of hospital settings without the need for costly instrumentation.

ID58. Rapid Molecular Detection of Influenza A and H275Y Mutation Conferring Resistance to Oseltamivir

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Introduction: Rapid molecular detection of influenza has become the standard for diagnosis and appropriate use of antiviral agents. However, the rapid emergence of oseltamivir-resistant seasonal H1N1 in the United States during the 2008-2009 influenza season revealed the additional need for direct detection of the nucleotide change causing resistance. We developed robust real-time amplification assays for influenza A and oseltamivir-resistant seasonal and pandemic influenza A viruses using redundant primer cocktails to withstand viral sequence drift. **Methods:** To reliably detect all subtypes of influenza A and withstand viral sequence drift, a highly conserved region within the M gene was targeted using a cocktail of primers that collectively provided 99.9% coverage of more than 9,200 sequences analyzed *in silico*. Similarly, oseltamivir resistance was detected via multiple forward primers specific to the seasonal and 2009 pandemic strains and a single reverse primer specific for the H275Y SNP of the neuraminidase gene. The assays include a spiked-in internal control (MS2 bacteriophage). Viral RNA was prepared from nasopharyngeal swab samples (easyMag, Biomerieux, France). The two-step single capillary RT-PCR assays using Plexor detection chemistry (Promega, Madison, WI) were performed in parallel using identical thermocycling conditions on the Roche LightCycler 2.0 (Indianapolis, IN). The assay was validated analytically using seven standard influenza isolates obtained from ATCC and CDC, and used to study 65 archived nasopharyngeal swabs from 2007-10 that had previously been examined using both Eragen (Madison WI) ASR influenza A/B reagents and a lab-developed assay for influenza A subtyping. **Results:** The MS2 target amplified in all retrospective samples. The pan-FluA assay was negative in 10 of 10 negative retrospective samples, and detected 52 of 55 samples positive by routine clinical PCR testing. Of these 52 FluA positive samples, the H275Y assay was negative in 9 archived seasonal H3N2 samples, in 10 seasonal H1N1 samples collected in 2007 (prior to the emergence of oseltamivir resistance), and 10 pandemic influenza H1N1 samples, and was positive in all but one seasonal H1N1 sample collected during the 2009-2010 influenza season, as expected. The three influenza positive samples that were negative using the pan-FluA assay, and the single H275Y negative 2010 seasonal H1N1 sample are under further investigation. **Conclusions:** We have designed and validated robust RT-PCR assays that utilize multiple primers to withstand sequence drift to detect influenza A and the H275Y SNP conferring resistance to oseltamivir in the N1 subtype, with results available in less than 45 minutes.

ID59. Detection and Identification of *Ehrlichia* and Other Vector-Borne Pathogens by PCR/ESI-MS

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Introduction: Ehrlichiosis presents with non-specific, flu-like symptoms, which makes accurate diagnosis difficult. *Ehrlichia chaffeensis* and *E. ewingii* are the two main species relating to different clinical outcomes in humans. Here we use a PCR electrospray ionization mass spectrometry (PCR/ESI)-based technique for the detection and identification of *Ehrlichia* species from whole blood. **Methods:** Specimens of whole blood were collected from patients with suspected tick–associated febrile illness. Nucleic acids from clinical specimens were extracted using a combination of mechanical and chemical lysis and magnetic bead purification. The specimens were then tested with a multi-locus assay utilizing 11 multiplexed primers targeting different loci that detect a wide range of vector-borne pathogens, including bacteria, protozoa, and viruses. ESI-MS of the PCR amplicons on an Abbott PLEX-ID was used to determine their base compositions that were subsequently used to identify the organisms found in the specimens. **Results:** In our study, specimens from patients with tick-associated febrile illness from the 2010 tick season in central Tennessee were analyzed. In 263 whole blood specimens tested, *E. chaffeensis* was detected in 18 (6.84%). *E. ewingii* was detected in 2 samples (0.76%). *Rickettsia rickettsii* and *Plasmodium vivax* were detected in one specimen each (0.38%). **Conclusions:** We demonstrated that our vector-borne microbial detection assay can detect pathogens from a whole blood using the same extraction method. This assay could provide physicians with actionable results for a broad range of pathogens, including unculturable species such as *Ehrlichia* and *Rickettsia* that would not have been detected by culture methods.

ID60. Characterization and Evaluation of a Novel Multiplexed Molecular Assay for the Detection of RSV and hMPV

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Introduction: Respiratory Syncytial Virus (RSV) and Human Metapneumovirus (hMPV) are significant respiratory pathogens of the very young and the elderly in both developed and developing countries. Further, these viruses present with the same symptoms during overlapping months, thereby confounding unaided clinical diagnosis. Molecular testing provides a sensitive and specific method for the detection and differentiation of these two upper respiratory pathogens. Utilizing proprietary chemistries and processes, we have developed novel room temperature stable reagents for use in our multiplex real-time RT-PCR assay for the detection of RSV and hMPV. This assay provides a simplified workflow and significantly reduces the number of steps required for testing. The objective of this study was to characterize the performance of the Quidel Molecular RSV+hMPV assay on 2 real-time PCR instruments and to evaluate this assay for the direct detection of leftover clinical specimens. **Methods:** RNA was extracted on the NucliSENS® easyMag® and 5 µl of each sample was added to reconstituted master mix and evaluated by real-time PCR. Preliminary performance characteristics of the RSV+hMPV assay, including analytical sensitivity, specificity and reactivity, precision and interference were determined. Extracted RNA and Kit stability parameters were also investigated. Testing on leftover patient specimens established the initial performance of this assay. **Results:** The Analytical Sensitivity of the RSV+hMPV assay was determined to be <5TCID50/mL for RSV and <55 TCID50/mL for hMPV. The RSV+hMPV assay detected RSV A, RSV B

and the 4 major sub-types of hMPV using reference virus strains. No cross reactivity or interference was observed with greater than 50 non-RSV and non-hMPV respiratory and non-respiratory viruses and other micro-organisms. Blood, mucin, and common prescription and over-the-counter cold, allergy and asthma medications studied did not interfere with the RSV+hMPV assay. Results from a 12 day inter-operator precision study showed that all positive samples had CV% values of <5% for all concentrations and conditions studied. Stability studies showed that extracted RNA was stable at all of the conditions studied. Leftover patient specimens were analyzed in the RSV+hMPV assay for the presence of either RSV or hMPV and were able to detect 23/23 RSV and 26/26 hMPV positive samples. Specificity was 100% for all samples evaluated. **Conclusions:** Results from these preliminary studies indicate that the RSV+hMPV assay is robust, simple to perform and sensitive and specific for the detection of RSV and hMPV.

ID61. Timing of the Appearance of HBV Mutants after HBV Nucleoside/Nucleotide Analogue Treatments by Analyzing with Massively Parallel Pyrosequencing Method

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Introduction: It is well known that the mutations of HBV polymerase are causative of the resistance during HBV nucleoside/nucleotide analogue treatment. Although it is considered that these mutations were appeared before DNA breakthrough, the definite time of their appearance is obscure. We studied for the timing of the appearance of mutant clones and their sequential change as analyzing serial serum samples from the chronic HBV patients experienced DNA breakthrough after nucleoside/nucleotide analogues treatment by massively parallel ultra-deep pyrosequencing method. **Methods:** Six patients with chronic HBV hepatitis who had DNA breakthrough after receiving the nucleoside/nucleotide analogue treatment at Eulji Medical Center in Korea were enrolled. Their serial serum samples were acquired with 3 or 6 month intervals during treatment. After DNA extraction, all samples were analyzed for HBV polymerase gene by massively parallel ultra-deep pyrosequencing using Roche GS FLX system **Results:** For 5 of 6 patients the small amount of drug-resistant mutations were observed before DNA breakthrough, at nadir state or before. The proportion of these mutant clones was variable with range of 1.0 to 24.2%. At DNA breakthrough the mutant clones had been dominant in all 6 patients. Other many kinds of mutations were also shown but their amounts were very small and not changed before and after breakthrough. **Conclusions:** It was identified that HBV mutations associated drug resistance were already present at nadir state. It was helpful for HBV treatment because early detection of mutant clones at nadir state can anticipate DNA breakthrough.

ID62. Comparison Between Two Methods for HCV Genotype Detection

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Introduction: Hepatitis C is an infectious disease that causes chronic liver disease such as cirrhosis, liver failure, or liver cancer. Hepatitis C virus (HCV) is an enveloped single-stranded positive sense RNA virus. The most common genotypes present in North America and Europe are 1 through 6 and their subtypes. HCV genotyping has been used to assess disease prognosis and the likelihood of therapeutic response, HCV genotyping can be used to identify the source of infection, particularly for geographically distinct subtypes. Determination of HCV genotypes has been made using a variety of technical methods, including line blot hybridization, RFLP analysis, real-time PCR, and direct nucleic acid sequencing. **Methods:** This study compared two different reagent kits and instrumentation configurations. The first method (M1) was ASR Versant HCV Genotype 2.0 Assay (LiPA) by Siemens Healthcare Diagnostics that was run on EasyMag, ABI 9700 thermal cycler, and Autoblot 3000. The LiPA assay is a PCR-based assay with detection utilizing line probe and reverse hybridization. The second method (M2) was Abbott RealTime HCV Genotype II RUO from Abbott Molecular that included m2000sp and m2000rt. The Abbott HCV assay is a real-time PCR method that utilizes MGB probes. Both methods were run using known HCV positive samples in plasma and serum matrix. **Results:** In the study, the following parameters were determined: workflow duration (M1 = 7hrs, M2 = 5hrs), hands-on time (M1 = 160min, M2 = 70min) LIS Interface (M1 = none, M2 = available), analytical performance (M1 = 1-6 genotypes, multiple subtypes, LOD 5000IU/mL and M2 = 1-6 genotypes, 1a and 1b subtypes, LOD 500IU/mL), sample tracking capability (M1 = manual, M2 = automated), laboratory contamination risk (M1 = high/open system, M2 = limited/closed system), assay regulatory status (M1 = ASR, M2 = RUO) and sample batch size (M1 = 18+2, M2 = 22+2). **Conclusions:** We concluded that both methods have comparable analytical and clinical utility. Siemens assay was able to detect more subtypes but requires a minimum viral load of 5000IU/mL. In comparison, Abbott assay detects 1a and 1b subtypes but needs a lower viral load of 500IU/mL. Advantages of using the Abbott system include shorter turnaround time, less hands-on time, interfacing capability, automatic specimen tracking, significantly reduce the risk of contamination, and finally analysis software standardizes analysis and interpretation process.

ID63. Comparison of Efficiencies for HR-HPV Detection of Hybrid Capture 2 and COBAS4800 in High Grade Lesions

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Introduction: The causal relationship between persistent high-risk human papillomavirus (HR-HPV) infection and cervical cancer is widely accepted. In addition, recent findings support theory that also substantial part of dysplastic lesions of anogenital area, e.g. vagina, anus, or penis, is connected in etiology with persistent HR-HPV infection. Nowadays, HR-HPV DNA testing either in triaging cytological diagnosis or in primary screening is a helpful tool in cervical cancer prevention. Characteristics and efficiency of the longest FDA approved and most common commercial method for HR-HPV DNA testing, Hybrid Capture 2 HR-HPV DNA test (HC2), have been verified in thousands of studies. HC2 is a sandwich capture molecular hybridization assay utilizing whole genome probe detecting 13 types of HR-HPV. Recently, new FDA- approved assays have arisen on the market. Some of them are PCR based targeting L1 region of HPV genome. However, during the process of HPV integration into human genome parts of HPV genome could be lost. As this process is likely to occur in high grade lesions, it might result in false negative finding of PCR based HR-HPV DNA test there. To assess the risk of such a false negative finding, we focused on comparison of testing efficiency of PCR based assay Cobas4800 (Roche) and HR-HPV DNA HC2 (Digene, Qiagen) in samples with high grade lesion. **Methods:** 90 samples including negative and positive controls were tested by both, Cobas4800 and HC2 assays. Discrepant samples were further analyzed by sets of PCR methods targeting more genes in HR HPV types. **Results:** We compared results of analysis of patient's samples, and positive and negative controls obtained by both methods, e.g. HC2 and Cobas4800. As expected, HPV cell lines controls known to be lacking the L1 region of HPV genome has been marked negative by Cobas4800 and positive by HC2. Furthermore, we have revealed discrepancy in 5 patient's samples. Genotyping assays have shown Cobas4800 missing HPV in 4 samples, and 1 case of false positive HPV finding by HC2. **Conclusions:** L1 deletion of HR-HPV may be quite frequent process in progression towards high grade lesions in anogenital area, particularly in cervical cancer. In countries with well organized long term cervical cancer screening only few patients could be missed by HPV testing targeting L1 gene of HPV. However, in other countries, more patients with high grade lesion are present in screening population and therefore endangered by false negative finding in L1 targeting PCR screening test.

ID64. Evaluation of the Gen-Probe APTIMA *Trichomonas vaginalis* Assay with the APTIMA Combo 2 Assay for Detection of *Chlamydia trachomatis* (CT) and/or *Neisseria gonorrhoeae* (GC)

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Introduction: The APTIMA *Trichomonas vaginalis* Assay is an in vitro qualitative nucleic acid amplification test (NAAT) for the detection of rRNA from *T. vaginalis* to aid in the diagnosis of trichomoniasis using the TIGRIS DTS System. The assay is approved to be used to test for *T. vaginalis* in the following specimens from symptomatic or asymptomatic women: i) clinician-collected endocervical swabs, ii) clinician-collected vaginal swabs, iii) female urine specimens, and iv) PreservCyt Solution liquid Pap specimens (i.e. ThinPrep). **Methods:** This study evaluated the accuracy of the APTIMA *Trichomonas vaginalis* Assay and studied the clinical usefulness of testing for *T. vaginalis* in patients co-infected with CT or GC. **Results:** There was 100% agreement when the accuracy of the APTIMA *Trichomonas vaginalis* Assay was assessed by testing 20 clinician-collected vaginal swabs (5 positive and 15 negative) and 27 female urine specimens (all positive). These specimens were all previously evaluated using the APTIMA *Trichomonas vaginalis* Assay through interlaboratory exchange. Ninety-four patients (72 female and 22 male) with a mean age of 23.7 yr that were determined to be positive for CT (90 patients) or GC (4 patients) with the APTIMA Combo 2 Assay were also tested for the presence of *T. vaginalis* in urine, vaginal specimens, ThinPrep, and penile specimens. Two of the 94 patients (2%) were also positive for *T. vaginalis*. Seventeen of the 92 specimens that tested negative for *T. vaginalis* were independently confirmed to be true negatives with the BD Affirm VPIII Microbial Identification Test that uses DNA probes. Three patients with GC or CT were determined to also have HSV-1 or HSV-2 by PCR or viral culture. **Conclusions:** The APTIMA *Trichomonas vaginalis* Assay is an accurate test and can easily be added to the testing of patients with the APTIMA Combo 2 Assay for GC and CT. In our study of 94 patients positive for GC or CT, 2% were co-infected with *T. vaginalis*.

ID65. Software Guided Assay Validation: Use of EZValidation™ in the Determination of the Performance Characteristics of a Real-Time PCR Assay Using MultiCode®-RTx for BKV Quantification

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Introduction: The experimental design and execution of studies to define performance characteristics of nucleic acid tests for pathogen nucleic acid quantification (qNATs) can be challenging. The aim of this study was to determine the utility of assay validation software (EZValidation, Life Technologies) while defining the performance characteristics of an assay for quantification of BKV DNA using MultiCode-RTx analyte-specific reagents (ASR, Eragen). **Methods:** The validation study was guided by the software and was comprised of design, definition, execution (results), analysis, and approval phases. The analyte, published assay validation "guideline," and instrumentation are entered in the design phase. The validation was performed using QIAsymphonySP extractor and ABI 7500 real-time PCR system. Acrometrix BKV panels were used as assay calibrators and test material. In the definition phase, accuracy, linearity, limit of detection (LOD), repeatability and reproducibility experiments were planned. Results were imported via electronic spreadsheet, analyzed by software, and determined to be acceptable as per user-defined criteria. The software did not support a quantitative comparison study using patient plasma specimens and a second real-time PCR assay; this experiment was performed separately using residual samples between 3.0-6.0 log10 copies/ml as quantified by an artus RUO-based assay. **Results:** Accuracy data fell within acceptable range of 0.3 log10 copy/mL difference (expected vs. observed). Linearity characteristics (slope delta < 0.1, intercept delta < 0.5, and R2 delta <0.1) also passed. LOD was estimated at 65 copies/mL (95% confidence interval, 48-88) using probit regression. Acceptance criteria for repeatability and reproducibility (<40% CV) were met at the three tested levels (3.7 log10, 4.5 log10, and 5.0 log10 copies/mL). The study summary including documentation of all phases was available as a printable report. **Conclusions:** Validation software provided an easy to use format for designing, organizing, executing, analyzing, and approving the results of validation studies. This could therefore be very useful for laboratories with little experience in assay validation. The data suggest that the MultiCode-RTx ASR-based assay has performance characteristics that are acceptable for quantification of BKV in clinical samples. One disadvantage that was encountered was the lack of sufficient software flexibility to enable study modification once experiments were designed and defined; correlation studies are also not presently supported.

ID66. Adaptation of the BD-Geneohm Cdiff Assay: Implementation of Automated Nucleic Acid Extraction from Stool Using the Nordiag Arrow Instrument

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Introduction: *Clostridium difficile* is a major cause of diarrhea in patients with a history of recent antibiotic treatment and hospitalization; most disease is caused by bacteria expressing the toxin B gene (*tcdB*). The gold standard detection method is toxigenic culture, a laborious and lengthy test. Real-time PCR assays that amplify *tcdB* have been increasingly adopted to improve the diagnostic utility of testing. The goal of this study was to determine the effectiveness of automated nucleic extraction of stool using the Nordiag Arrow instrument for use with the BD-Geneohm Cdiff assay, a real-time PCR test FDA cleared for use with manually extracted nucleic acid. **Methods:** Randomly selected stool samples (n= 378) that had been tested by standard BD-Geneohm method were de-identified after the initial real-time PCR result was recorded. 0.25 mL (~30 mg) of stool were dissolved in 1 mL proprietary stool stabilizer buffer, vortexed, incubated for 1 min at room temperature, then centrifuged for 5 minutes at 6000 rpm. 0.5 mL of dissolved stool was extracted using the Stool DNA 039 protocol and DNA was eluted in 0.1 mL. Three microliters of eluate were used in the BD-Geneohm Cdiff assay as per standard protocol. Discrepant results were adjudicated with toxigenic culture. The institutional prevalence of 15% was used for statistical analysis; calculations were performed with SISA (*http://www.quantitativeskills.com/sisa/statistics/diagnos.htm*). **Results:** Compared to the FDA cleared method, the modified method had the following performance characteristics (95% confidence intervals): overall agreement, 98.1% (96.4%-99.9%); kappa, 0.93 (0.8-1.06); positive percent agreement, 93.1% (84.8%-101%); negative percent agreement 99.1% (98%-100%); positive diagnostic likelihood ratio, 99.3 (23-418); negative diagnostic likelihood ratio, 0.07 (0.02-0.23). Seven discordant results were observed (3 positive by Arrow but negative by manual extraction, 4 negative by Arrow but positive by manual extraction); results of discordant analysis are pending. **Conclusions:** Automated extraction on the Nordiag Arrow is a suitable replacement for manual extraction of stool for use with the BD-Genohm Cdiff assay.

ID67. Evaluation of APTIMA *Trichomonas vaginalis* Assay

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Introduction: *Trichomonas vaginalis* (TV) is one of agents causing sexually transmitted disease (STD). Detection of TV with the traditional method such as wet-mount preparation (wet prep) has been used in clinical setting. *T. vaginalis* APTIMA assay is a nucleic acid amplification method (NAA) that can potential used in the same way for detection of *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT). This study is to evaluate if TV can be detected by NAA in the same way as for detection of NG and CT. **Methods:** 484 randomly selected clinical samples sent for NAA by APTIMA TMA (Gen-Probe) for *N. gonorrhoeae* and *C. trachomatis* were used for the prevalence study of *T. vaginalis*. The results of wet prep and NAA by APTIMA (Gen-Probe) for TV were used for assay validation. **Results:** Of 484 samples tested, 107 (22.1%) were positive for TV and 377 (77.9%) were negative for TV. Among 484 samples tested, 21 were positive for NG, 66 for CT, and 22 for NG and CT, thus total of 109 (23%) were positive for NG or CT. Of 107 tested positive for TV, there were 24 samples tested positive by NAA test for NG or CT (1 NG, 16 CT, and 7 NG and CT). There were 125 samples that had the wet prep test ordered in addition to the NAA for NG and CT. Among 125 samples tested, 54 were tested positive by APTIMA for TV, 71 were negative for TV. Among 54 APTIMA-positive samples, 37 were positive by wet prep. Thus the sensitivity of wet prep for TV could be 68.5% among those samples. Among 37 wet-prep and APTIMA confirmed samples for TV, one sample was positive for NG, 6 for CT, and 1 for NG and CT. There were 8 samples (21.6%) tested positive for infection with TV, NG or CT. Among 71 APTIMA-negative samples, 2 were reported positive by wet prep. Thus the false positive rate of NAA could be 2.8%. Among 65 TV-negative samples by both wet-prep and APTIMA, 4 were positive for NG, 10 for CT, and 4 for NG and CT. There were 18 samples (27.7%) tested positive for NG or CT. **Conclusions:** The preliminary data demonstrate the need and capability of NAA test by APTIMA in detection of *T. vaginalis* along with the detection of *N. gonorrhoeae* and *C. trachomatis*.

OTHER (EDUCATION, ETC.)

OTH02. The Rapid Expansion of a Molecular Pathology Laboratory

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Introduction: As our knowledge of the molecular mechanisms underlying human disease continues to increase, the transition of this knowledge into the clinical setting becomes critical to patient management. Pathology departments must be prepared to meet the increasing demands for new molecular based testing that has become standard of care. Our department committed to the creation of a core molecular facility to perform molecular testing across many disciplines. Here we demonstrate the effective and rapid expansion of the laboratory to meet the needs of the clinicians and combat the rising cost of reference lab testing. **Methods:** In 2008, Baystate Health committed to expanding the operating space and budget of their small and limited molecular pathology laboratory. This included the design and creation of a laboratory suited to the unique needs of molecular testing. In addition, new equipment was selected that would optimize technical resources and allow for rapid expansion of a test menu. Training, re-distribution of workload, streamlining of workflow, and coding/billing were all consider as part of this process. **Results:** The purchase of innovative variable platforms gave the laboratory the ability to provide a variety of different tests across different medical disciplines. The introduction of the AB 7500 Fast real-time PCR instrument, AB 3130 Genetic Analyzer, Roche COBAS Ampliprep/COBAS Taqman, and the Luminex 200 gave the laboratory significant flexibility and endless opportunities to maximize their use. A rapid expansion of the test menu from 5 tests to 15 tests was achieved with a team of 7 technologists, all of whom re-trained on new instruments and validation processes. **Conclusions:** A combination of FDA cleared assays and LDT's were used to develop needed testing to support clinical needs in oncology, virology, inherited disease and pharmacogenomics. Eighteen months after the doors opened, the core molecular laboratory has been able to add 10 tests to its menu and increase its volume by more than 8,000 tests annually.

SOLID TUMORS

ST01. Progression to Esophageal Adenocarcinoma in Barrett's Esophagus Patients with a Polysomy versus Nonpolysomy Fluorescence *in Situ* Hybridization (FISH) Result

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Introduction: Identification of patients with Barrett's esophagus (BE) who will progress to esophageal adenocarcinoma (EA) is a challenge. Fluorescence *in situ* hybridization (FISH) has been shown to be useful for the detection of dysplasia and EA in endoscopic brushing specimens from patients with BE. The aim of this study was to determine if FISH can be used to predict which high-risk BE patients are most likely to develop EA. **Methods:** Endoscopic esophageal brushing specimens from 281 high-risk BE patients (86% had a history of high-grade dysplasia) without a previous diagnosis of EA (240 males and 41 females, median age of 66 years, range 22-90 years) were collected in conjunction with surveillance biopsy (four quadrant biopsies every centimeter of affected mucosa). Specimens were examined with FISH probes to 8q24 (*MYC*), 9p21 (*CDKN2A*), 17q12 (ERBB2), and 20q13.2 (ZNF217) (Abbott Molecular Inc., Des Plaines, IL). Specimens exhibiting ≥4 polysomic cells (≥3 signals for ≥2 loci) were considered polysomic while specimens with all other genetic findings (e.g. 9p21 loss, single locus gain, disomy/normal) were placed into the non-polysomic group for statistical analysis. Follow-up intervals (median 29.0 months) for progression to EA were calculated as the time between FISH and the first histologic diagnosis of EA or to the last endoscopy for patients who did not present with EA during follow-up surveillance. The risk of EA was compared between polysomy and non-polysomy groups with the logrank test and estimated using the Kaplan-Meier method. **Results:** Histology results at the time of FISH analysis included 117 (42%) high-grade dysplasia, 71 (25%) low-grade dysplasia, 53 (19%) intestinal metaplasia and 40 (14%) benign squamous epithelium. Twenty-one of the 281 (7.5%) patients were found to have EA on follow-up. Of the 98 patients (median follow-up 21.1 months) with a polysomic FISH result, 17% developed EA while 2% of the 183 patients (median 38.2 months) with a non-polysomic FISH result developed EA (p=0.0001, Logrank test). The estimated risk of developing EA in three years from baseline was 21.3% among patients with a polysomic FISH result (95% CI: 11.1% to 31.5%) as compared to 0.6% among patients with a non-polysomic FISH result (95% CI: 0% to 1.8%). **Conclusions:** These data indicate that patients with a polysomic FISH results are at greater risk for developing EA compared to those without polysomy. Further studies are needed, but these findings suggest that FISH could be used to modify the frequency of endoscopic surveillance for patients with BE.

ST02. *BRAF* Mutation Testing in Malignant Melanoma: Pitfalls of Allele-Specific PCR

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Introduction: BRAF is commonly deregulated in human malignancies, including colorectal cancers, papillary thyroid carcinoma, and malignant melanoma. The recent introduction of therapeutic agents targeting mutated *BRAF*, especially in melanoma, has increased the need for accurate diagnostic assays. A single nucleotide hotspot mutation (NM_004333:c.1799T>A, p.V600E) accounts for a large percentage of described mutations; however, in malignant melanoma other mutations in this region, including V600K, V600R, and V600D, constitute a significant minority of up to 20%. Commonly used commercial and home-brew clinical assays for *BRAF* mutations employ methods to detect the V600E mutation but may not correctly classify other *BRAF* mutations. We studied the performance of our home-brew *BRAF* V600E allele specific PCR (AS-PCR) assay on fine-needle aspirates (FNAs) of metastatic melanoma as compared to Sanger sequencing. **Methods:** Air dried Diff-Quik stained cytologic smears from 15 FNAs of metastatic melanoma were studied. Areas enriched with tumor cells were identified by a cytopathologist, microdissected, and tumor DNA was extracted. AS-PCR for *BRAF* V600E with capillary electrophoresis detection was performed. A control PCR reaction targeting *BRAF* exon 15 was performed in parallel for all samples. Sanger sequencing of *BRAF* exon 15 was also performed on all cases. **Results:** By AS-PCR, 14/15 samples yielded adequate amplification. 5/15 samples tested positive for the V600E mutation and 6/15 samples were wild type. In these cases, Sanger sequencing confirmed the V600E strong positive and wild type results; however, one V600E positive sample harbored an alternate V600E mutation (c.1799_1800delinsAA). 3/15 samples yielded weak AS-PCR products for V600E, which were much less than typically seen in V600E positive samples. On repeat analysis these peaks were consistently below or minimally above the threshold for a positive V600E interpretation. These three samples with small amplification peaks on V600E AS-PCR demonstrated V600K mutations (c.1798_1799delinsAA) by sequencing. **Conclusions:** Allele-specific PCR detects *BRAF* V600E mutations in malignant melanoma; however, variant *BRAF* mutations commonly found in melanoma, such as V600K, can confound assay interpretation leading to inaccurate test reporting. In our series, 4/8 *BRAF* mutant melanomas harbored variant *BRAF* mutations that, depending on assay design, may be inaccurately detected or not detected at all. The correct distinction and reporting of variant *BRAF* mutations is critical for trials that are evaluating *BRAF*-targeted therapies in melanoma.

ST03. Implication of *BRAF* V600E Mutation Status for Prognosis in Colorectal Cancer

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Introduction: *BRAF* V600E mutations are frequently found in several cancer types including melanoma and thyroid carcinomas. This mutation can also be found in a smaller percentage of colorectal cancers. Although this mutation is found in 10% of colorectal cancers, recent evidence highlights the potential prognostic value of *BRAF* gene alteration in patients with colorectal cancers. In this study, we assessed the impact of *BRAF* V600E mutation on patient survival in *KRAS* negative colorectal cancers. **Methods:** DNA was extracted from 25 FFPE tumor tissues (one 10 µm section) from patients with colorectal cancer treated with resection and chemotherapy. Percentages of tumor tissue in total tissue were estimated for each block and ranged from 15% to 70%. Each DNA sample was previously tested with *KRAS* mutation specific TaqMan assays and now subjected to a SYBR Green real-time PCR assay consisting of separate amplifications with wild-type and V600E-specific primers. Amplification curves of the wild-type and mutant PCRs were compared using delta CT analysis and compared to a 1% mutant control used to set the limit of detection for the assay. For each test run, NTC, negative and positive controls gave the expected results. **Results:** The 25 tumors were previously genotyped as *KRAS* wild type. Of these 25 tumors, 18 (72%) were also *BRAF* wild type and 7 (28%) harbored the *BRAF* V600E mutation. The *BRAF* wild-type patients were treated between 2003-2006, while the patients with *BRAF* V600E mutations were treated between 2007 and 2010. Of the 18 *BRAF* wild-type patients, 4 (18%) patients were deceased, while 4/7 (57%) of the *BRAF* mutant patients were deceased. The mean survival of the *BRAF* wild-type patients was approximately 6 years, while the survival of patients with *BRAF* V600E mutations was 1-2 years. The surviving *BRAF* V600E mutated patients all had metastatic disease detected within less than 2 years of diagnosis. In all patients with *BRAF* V600E mutations, the primary tumors were located in the right colon. **Conclusions:** Our data show that *BRAF* V600E mutation status appears to be associated with poorer outcomes in colorectal cancer patients, suggesting that *BRAF* mutation analysis should be included in the prognostic assessment of these patients. In addition, our findings suggest that *BRAF* mutation testing in patients with right-sided colon cancer may be useful in identifying individuals with a worse prognosis who would benefit from individualized therapy.

ST04. qPCR Analysis of MicroRNA Expression Differences in Formalin-Fixed, Paraffin-Embedded Pancreatic Ductal Adenocarcinoma, Intraductal Papillary Mucinous Neoplasm, and Pancreatitis Tissues

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Introduction: Pancreatic ductal adenocarcinoma (PDA) is an aggressive disease with low survival rates. Diagnosis can be difficult, as it may resemble pancreatitis both radiologically and histologically. Previous studies have demonstrated differential expression of microRNAs in these diseases as compared to normal pancreas (NP). The goal of this study was to differentiate pancreatic adenocarcinoma, intraductal papillary mucinous neoplasms (IPMN), and pancreatitis on the basis of microRNA qPCR of formalin-fixed, paraffin-embedded (FFPE) tissue cores, as histologic diagnoses are often made utilizing tissue cores from fine needle aspirations. **Methods:** Tissue cores were obtained from FFPE tissue blocks from pancreatectomy specimens carrying diagnoses of PDA, IPMN, and pancreatitis. After re-embedding, sections were used for total RNA (including miRNA) isolation (FFPE RNA Purification Kit, Norgen Biotek Corp., Thorold, ON, Canada). MiRNA expression levels for 10 miRNA were analyzed using qPCR (Taqman microRNA assay, Applied Biosystems, Carlsbad, CA). Statistical analysis was performed using ΔCt values normalized to miR-16, which was previously shown to have consistent expression among pancreatic tissues types, rather than rRNA. A total of 27 PDA and adjacent NP, 10 IPMN and adjacent NP, and 10

pancreatitis samples were evaluated. **Results:** Analysis of miRNA expression levels yielded multiple miRNA with discriminatory abilities. MiR-21, -24, -155, and -203 were significantly differentially expressed based upon the histology of the sample, and discriminated between pancreatitis, IPMN, and pancreatic cancer. Mir-191, -301, -210, -221, -375 and -376a showed no discriminatory capabilities. Reduced mir-24 expression alone was able to effectively discriminate pancreatitis from IPMN, PDA, and normal tissues. Significantly increased expression of mir-155 was seen in PDA compared to adjacent normal tissue. There was increased expression of mir-21 in all disease states compared to normal pancreatic tissues. Mir-203 showed significantly increased expression in PDA. **Conclusions:** qPCR of microRNAs has potential as an ancillary test in differentiating pancreatic lesions. From a set of 10 miRNA targets, a subset of 4 (miR-21, -24, -155, and -203) could be used to discriminate the majority of PDA, IPMN, and pancreatitis. The use of a single miRNA (miR-24) was able to discriminate pancreatitis from neoplasia (IPMN and PDA). Further testing using an expanded sample set is underway to fully demonstrate the utility of miRNA quantitation in assessment of pancreatic lesions.

ST05. A Novel Internal Tandem Duplication of *KIT* Exon 11 Confers Responsiveness to Imatinib

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Introduction: *KIT*, a proto-oncogene located on human chromosome 4q, codes for a type III receptor tyrosine kinase critical for development of multiple cell types. *KIT* activating mutations have been identified in various malignancies, including gastrointestinal stromal tumors (GISTs) and approximately 2% to 3% of malignant melanomas (MM) (most commonly the acral, mucosal and ocular subtypes). Greater than 95% of melanoma *KIT* mutations are found within exons 9, 11, 13 and 17. In contrast to exon 11 insertion/deletion mutations commonly found in GISTs, MM most commonly shows point mutations or small deletions. However, the full spectrum of mutations in MM is unclear, and data is only beginning to emerge regarding which melanoma mutations are susceptible to *KIT*-targeted therapy. **Methods:** 76 cases of MM seen at the Hospital of the University of Pennsylvania since 2010 (all confirmed negative for the *BRAF* V600E mutation) were reviewed by staff surgical pathologists. Selected tumor-enriched areas were macrodissected and genomic DNA was isolated. Exons 9, 11, 13, and 17 of *KIT* were amplified by PCR and labeled using BigDye Terminator v3.1/Xterminator reagents (ABI). Samples were electrophoresed using a 3130xl Genetic Analyzer (ABI) and analyzed using Mutation Surveyor V3.97 software (SoftGenetics). Confirmatory testing was performed at a reference laboratory. **Results:** *KIT* activating mutations were seen in 8 of 78 cases of MM (10.2%), with mutations most frequent in acral (3/8) and mucosal (2/8) melanomas as previously described. Mutations were observed in exon 11 (6/8) and exon 17 (2/8). Missense mutations comprised 7/8 cases. One patient with metastatic acral melanoma showed a novel 54 bp internal tandem duplication (ITD) mutation within exon 11, resulting in an 18 amino acid insertion in the juxtamembrane domain. **Conclusions:** *KIT* mutations in melanoma occurred most frequently in exon 11 and consisted mainly of point mutations, as previously described. Missense mutations ranged from common (L576P, 3 cases) to relatively rare (N822Y, one case). A novel 54 bp exon 11 ITD was found in a patient with acral melanoma who showed an excellent response to imatinib, with resolution of inguinal and pulmonary metastases and lack of progression following 8 months of treatment. One other patient with vulvar mucosal melanoma (L576P mutation) shows stable disease after 9 months of dasatinib treatment. Our findings highlight the lack of complete knowledge regarding the spectrum of *KIT* mutations in melanoma, and suggest that *KIT* mutational assays for melanoma should not be designed to target only particular classes of mutations.

ST06. *IDH1* Mutation Is a Favorable Prognostic Factor for Type 2 Gliomatosis Cerebri

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Introduction: Recent studies have demonstrated the prognostic significance of *IDH1* mutations in glioma. However, it is unclear whether *IDH1* mutation is also a prognostic factor in gliomatosis cerebri (GC). Primary GCs can be grouped into Type 1 GCs, which represent classical lesions with diffuse neoplastic growth without mass formation, and Type 2 GCs, which have obvious neoplastic masses in addition to diffuse lesions. Type 1 and Type 2 GCs are thought to have different molecular features and prognostic factors. **Methods:** Here we evaluated the prognostic relevance of *IDH1* mutation on overall survival (OS) and progression-free survival (PFS) in 74 GCs including 43 Type 1 and 31 Type 2 GCs. *IDH1* and *IDH2* alterations of the mutational hotspot codons R132 and R172 were assessed by bidirectional Sanger sequencing and PNA-mediated clamping PCR. **Results:** We detected 33 *IDH1* mutations in 74 GCs (44.6%), and no *IDH2* mutations. The percentage of two-year OS for wild-type *IDH1* patients was 46% versus 72% for patients with *IDH1*-mutated tumors. Multivariate analyses revealed that *IDH1* mutation was an independent favorable prognostic factor predicting OS in GC. In further analyses according to growth type, *IDH1* mutation was strongly correlated with favorable prognostic outcomes of Type 2 GCs, but not of Type 1 GCs. **Conclusions:** *IDH1* mutation may be useful prognostic factor in GC, especially for Type 2 GC. In addition, Type 1 and 2 GCs appear to have different molecular features.

ST07. Quality Assessment of Lung Fine Needle Aspirates (FNA) for Mutational Analysis

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Introduction: Personalized therapy based on tumor molecular testing is evolving rapidly. The need to provide molecular results quickly with limited specimen material is becoming a constant challenge. At our institution, computed tomography (CT)-guided fine-needle aspirations (FNA) are routinely used for diagnostic assessment of lung tumors. In order to guide patient therapy, mutation results for *EGFR* and *KRAS* are often requested on this limited specimen type. In this study we evaluated the utility of lung Fine Needle Aspirates (FNAs) for mutational analysis when compared with routinely performed testing of surgically resected specimens. **Methods:** 101 lung formalin-fixed FNA specimens were used for molecular analysis. At least 50% of tumor cells were required to be present in the specimen. After manual microdissection and DNA extraction, optical density (OD 260/280) readings were obtained and DNA concentration was measured using the Nanodrop 1000. Detection of *EGFR* exons 19 and 21 and *KRAS* codons 12 and 13 and was performed with Sanger sequencing. Results of FNA analysis was compared to mutational analysis of 1467 surgically removed FFPE lung tumor tissues tested for the presence of mutations from Jan 2007 to April 2011 at the University of Pittsburgh Medical Center. **Results:** In lung FNA specimens, 93% of samples were informative for *KRAS* and *EGFR* mutational analysis, while 7% (7/101) demonstrated absence or low level of PCR amplification and were considered as indeterminate for molecular testing. The average DNA concentration for indeterminate FNAs was low (1.7 ng/μl, range=0.2-4.4 ng/μl) as compared to cases informative for analysis (8.5 ng/μl, range=1.5-27.5 ng/μl). In addition, in 100% (7/7) indeterminate cases with low concentration of DNA had OD ratio measurements out of acceptable range (1.8-2.0). In surgical specimens, only 2% (25/1467) were indeterminate for molecular analysis. Interestingly, in these indeterminate surgical specimens, the quantity of DNA was acceptable for molecular testing (average: 10.8 ng/μl), however OD 260/280 readings were out of range in 60% of cases. *EGFR* and *KRAS* mutations were detected in 30% of FNAs and 25% of resected cases. **Conclusions:** Lung FNA samples were informative for mutational analysis using Sanger sequencing in 93% of cases and demonstrated a mutation detection rate similar to resected tumor specimens. For all specimens indeterminate for molecular analysis, OD ratio, 260/280, was a better predictor of the quality of PCR amplification as compared to DNA concentration.

ST08. Heterogeneous Staining for DNA Mismatch Repair Proteins Encountered During Lynch Syndrome Tumor Screening: A Somatic Phenomenon?

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Introduction: Immunohistochemical staining (IHC) for DNA mismatch repair (MMR) protein expression is used alone or in combination with microsatellite instability testing (MSI) in tumor screening for Lynch Syndrome. Abnormal screen results often prompt further tumor testing (*MLH1* promoter methylation, *BRAF* mutation analysis) or germline sequencing of MMR genes to differentiate between Lynch Syndrome and sporadic MSI-high (MSH-H) tumors. While IHC interpretation is usually straightforward, rare tumors display unequivocal heterogeneity of expression for one or more MMR proteins, the significance of which is uncertain. We describe a series of such cases. **Methods:** Reports and IHC slides were reviewed to find examples of heterogeneous IHC staining. Heterogeneous loss (HL) was defined as areas of loss of DNA MMR staining in tumor nuclei in contrast to positive staining of internal controls (i.e., stroma, lymphocytes) and positive nuclear staining in separate areas of the same tumor. **Results:** Twenty cases showed HL of one or more MMR proteins. Eleven were colorectal adenocarcinomas and 9 were endometrioid-type endometrial adenocarcinomas. The most common staining pattern in the cohort was HL of *MLH1* and *PMS2* with normal staining for *MSH2* and *MSH6* (n=11). The second most common pattern was *MSH6* HL with complete loss (CL) of *MLH1* and/or *PMS2* (n=7). Of the 11 cases showing HL of *MLH1*, 5 tested cases exhibited *MLH1* promoter methylation and 1 did not. Of 16 cases tested for

microsatellite instability, 14 were MSI-H and 2 were microsatellite stable (MSS). The two MSS cases exhibited *MLH1* promoter methylation and were in the *MLH1* HL group. Only 2 patients had follow-up germline testing (*MLH1*), and a mutation or deletion was not detected. **Conclusions:** The significance of heterogeneous MMR staining in tumors remains unclear; however, it seems likely that it represents a somatic event since it is not present in all tumor cells. The finding of *MLH1* promoter methylation in some of the cases provides support for this contention. HL of DNA MMR protein expression may lead to discordant MSI testing results if the area of tumor used for MSI testing is different than the area of tumor that shows loss of MMR expression. Reporting of HL is probably warranted both to explain discordant MSI results and to direct potential germline testing until a more systematic study can prove the somatic nature of the finding.

ST09. Verification of a Laboratory Developed Assay (LDA) for Detection of *KRAS* and *BRAF* Mutations from Formalin-Fixed Paraffin-Embedded (FFPE) Tumor Tissues

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Introduction: Identification of allele-specific mutations in tumor samples from FFPE tissues has increasingly become important in the clinical management of cancer due to development of gene-specific anti-cancer therapies. Here, we report the clinical verification of a laboratory developed real-time PCR *KRAS*/*BRAF* mutation panel using commercially-available research use only reagents (EntroGen Inc., Tarzana, CA). **Methods:** Tumor samples from thirty-one patients (12, lung cancer; 11, melanoma; 6, colon cancer; and 2, not specified) were retrospectively collected and compared to results obtained from reference laboratories (Clarient, Genzyme, and ARUP) with 9 positive for *KRAS* mutations, 3 positive for *BRAF* V600E and 20 negative for *KRAS* and *BRAF* mutations. The LDA consists of 12 individual real-time PCR assays designed to detect *KRAS* mutations on codons 12, 13, and 61 and the *BRAF* mutation V600E on codon 599. FFPE tissue samples were collected in two ways from FFPE blocks: three 10 micron sections, or 1.5 mm circular cores. The core method was used on solid tumor samples to preserve sample in the tissue block for future use. **Results:** The percent positive and negative agreements between the LDA and reference laboratory assay results were both 100%. The *KRAS* and *BRAF* LDAs were found to detect 2.5% and 20% of mutant DNA in the presence of wild-type DNA extracted from FFPE cell pellets, respectively. The inter-run coefficients of variation of the LDA cycle threshold values for *KRAS* G12V and the *BRAF* V600E mutations were 0.46% and 7.8%, respectively. The core method was found to be comparable to sectioning in concentration and quality of extracted DNA, but simpler and less labor intensive. **Conclusions:** The LDA was accurate, reproducible, and robust for detection of *KRAS* mutations in lung and colorectal cancers and the identification of *BRAF* V600E in colorectal cancers and melanoma. Local availability of molecular oncology tests can reduce turn-around-times for results, save costs, and provide for better stewardship of often limited tumor tissue.

ST10. Design and Initial Validation of a Next-Generation Sequencing-Based Assay for Detecting Somatic Mutations in Clinically Actionable Cancer Genes

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Introduction: Molecular testing is becoming standard-of-care for some cancers because certain mutations provide information about prognosis and treatment. Current methods for mutation detection are only capable of interrogating selected regions of individual genes, which is inefficient when testing multiple genes is clinically indicated. We are conducting a pilot study using Agilent solution-based genomic enrichment and Illumina sequencing for comprehensive mutation analysis of multiple cancer genes in a single assay. We have designed and completed initial validation for an assay that targets 28 genes frequently mutated in solid and hematologic malignancies. **Methods:** Baits for Agilent solution-based capture were designed to target exons +/- 200bp and +/- 1000bp of each gene. Initial validation was performed using DNA from three HapMap individuals with known single nucleotide polymorphism (SNP) genotypes, and DNA derived from frozen and formalin-fixed specimens from two lung adenocarcinoma tumors, also with previously-generated SNP array data. Multiplex Illumina sequencing of validation samples was performed following library construction and Agilent solution-based capture. Reads were mapped using novoalign and sequence differences were identified and annotated using samtools and a custom analysis pipeline. Sensitivity and specificity for detecting single-nucleotide variants was assessed by comparing sequencing basecalls to array-based SNP genotypes. **Results:** The assay was designed to target 437,694 base-pairs across the 28 cancer genes in our panel, although some positions were not covered by capture baits because of repetitive sequence. Hybridization capture and multiplex sequencing of initial validation samples produced 4-6 million reads per sample, of which ~70% were unique reads that mapped to the target region. Average coverage depth across all target regions was 800- to 1000-fold per sample. Read count, on-target percentage and error rates were similar between the three sample types. Consensus basecalls from the seven validation samples were 96% concordant with array-based genotypes at 106 sites for the tumor samples and 344 sites for the HapMap samples. In addition, a missense mutation was identified in the *KRAS* gene in one of the tumor samples at a position known to be mutated in non small cell cancer. **Conclusions:** Initial validation of a targeted next-generation sequencing-based assay for somatic mutations revealed excellent enrichment for targeted regions and accurate identification of previously-known genotypes in seven samples. Additional validation is necessary to characterize the performance of this assay, but our preliminary studies suggest it will be an efficient alternative to established methods for detecting somatic mutations in cancer, particularly when testing multiple genes is indicated.

ST11. Deep Sequencing of Target Genes in Solid Tumors Reveals New Deletions and Insertions in Microsatellite Sequences

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Introduction: Multiple genetic changes occur in cancer cells, including activation of oncogenes and inactivation of tumor suppressor genes. Mutations that disrupt gene functions that are important to cell growth and development could contribute cancerous development. Similarly, deletions or insertions in coding regions can result in either deletion or insertion of amino acids or reading frame shift with a truncated protein. **Methods:** To identify such deletions and insertions, we selected 276 genes that had a high tendency to mutate in breast, colorectal, lung, or prostate tumors for deep sequencing. DNA BAITs of 120 nucleotides were designed to cover all exons of the target genes and corresponding RNA BAITs were generated for target enrichment. By coupling Illumina GA II sequencing system with multiplexing and Agilent SureSelect target enrichment system, we achieved ~ 60x coverage deep sequencing on the exons of the target genes in 29 breast, 22 colorectal, 22 lung and 7 prostate tumors. After assembling paired sequence reads to human genome, small deletions and insertions were detected in the samples. After a series of filtering steps and computational analyses, followed by visual examination of sequence assembly at the mutation sites, deletions and insertions in these tumors were identified. **Results:** Ten deletions and insertions were identified and confirmed by Sanger sequencing. Among them, 4 cause reading frame shift, 5 cause 1-6 amino acids deletion, and 1 results in 2-amino acid insertion. Most of the mutated genes are known to play important roles in tumor development. Interestingly, 8 of the 10 mutations occur in microsatellite sequences. These mutations either increase or decrease the number of the repetitive units and are believed the products of microsatellite instability (MSI) in tumors due to impaired mismatch repair system. In addition, 7 of the microsatellite mutations were detected in colorectal cancers, and 4 are detected in colorectal cancers only. **Conclusions:** This study indicates that MSI in sporadic colorectal cancers may be more prevalent than currently believed 15%. It also suggests that MSI is present in breast, lung and prostate cancers, to less extent, and could lead to identification of new diagnostic markers for cancer development and progression.

ST12. Oligonucleotide-Based *in Situ* Hybridization for the Detection of *EGFR* Variant III in Glioblastoma

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Introduction: The Epidermal growth factor receptor (*EGFR*) gene is commonly altered in a wide range of human tumors including Glioblastoma (GBM). *EGFR* variant III (*EGFR*vIII) is a common alteration in GBM, and results from in frame skipping of exons 2-7 of the wild-type EGF receptor. This is often coupled to amplification of the *EGFR* locus. In this study we developed an oligonucleotide based mRNA *in situ* hybridization (ISH) assay to detect both wild type and vIII mRNA expression status of the *EGFR* gene in previously diagnosed GBM patients. **Methods:** 15 GBM formalin-fixed, paraffin-embedded (FFPE) tissue blocks were selected from the records of the Massachusetts General Hospital Department of Pathology based on *EGFR* fluorescence *in situ* hybridization (FISH) results. These included 8 cases with *EGFR* amplification and 7 cases with normal DNA copy numbers. An oligonucleotide based mRNA ISH assay was developed with two sets of probes. The first set detects normal *EGFR* expression with oligonucleotide probes in exons 2-7. The second set detects *EGFR*vIII expression using oligonucleotide probes that recognize the exon 1 and 8 fusion sequence after skipping of exons 2-7 (Quantigene View RNA ISH, Affymetrix, CA). *EGFR*vIII RT-PCR and oligonucleotide lysate detection (QG 2.0 lysate assay, Affymetrix, CA) were also performed using RNA extracted from the same cases as gold standards. **Results:** 5 out of 8 *EGFR* amplified GBM cases (62.5%) showed *EGFR*vIII mRNA expression with the developed QuantiGene View RNA ISH Assay. The signals were strong, cytoplasmic,

discrete and specific to tumor cells. None of the GBM cases with normal *EGFR* copy number showed vIII expression. Oligonucleotide lysate detection confirmed the same results. *EGFR*vIII RT-PCR applied to tissue lysates showed positive bands in 4 out of 5 detected positive cases. Our internal control assay, GAPDH, showed very low level of signals in the 5th case, suggesting the sample with degraded RNA. Although RT-PCR was negative in this case, our assay, because of its oligo based nature and being in situ, was able to detect the signals in a small number of intact cells left in the tissue. **Conclusions:** We have developed an oligonucleotide-based mRNA ISH assay for *EGFR*vIII that performs comparably to RT-PCR in FFPE samples. Being oligo based and using multiple probes per target make this assay very sensitive and specific. Given the absence of specific antibodies for this aberration, this provides a unique tool to detect this aberration *in situ* in GBM and potentially other tumors.

ST13. Validation of a FISH Assay to Identify *JAZF1* Rearrangements in Primary and Metastatic Endometrial Stromal Tumors

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Introduction: Endometrial stromal sarcoma (ESS) is a rare uterine malignancy with the potential for aggressive behavior that frequently shows variable histologic and immunophenotypic features making diagnosis difficult. In addition, metastatic ESS often presents with a long latency (20 years or greater) or without a previously identified primary lesion. Endometrial stromal nodule (ESN) is histologically similar to ESS but without infiltrative growth into the myometrium. Also, high grade lesions previously included in the ESS diagnostic spectrum are now classified as the histologically distinct entity undifferentiated endometrial sarcoma (UES). Rearrangements of *JAZF1* at 7p15 resulting in fusion with partner genes *JJAZ1* at 17q11.2 or *PHF1* at 6p21 reportedly occur rarely in UES but are present in greater than 70% of classic ESS and ESN. Therefore, we developed a fluorescence *in situ* hybridization (FISH) assay using a break-apart strategy (BAP) to detect rearrangements of *JAZF1* to aid in the diagnosis of these tumors. **Methods:** Lab developed FISH probes were constructed using bacterial artificial chromosome (BAC) clones covering 658 kb adjacent to the 5' end of *JAZF1* (labeled in SpectrumGreen™) and 635 kb adjacent to the 3' end of *JAZF1* (labeled in SpectrumOrange™). The FISH probe was evaluated in a blinded fashion by two reviewers scoring 50 nuclei each (100 total). Thirty-seven paraffin-embedded tissue samples of primary or metastatic tumor from patients suspected or diagnosed with ESS, ESN, or UES and 26 normal endometrium/myometrium control specimens were tested. The specimens were processed and scored using standard culture and FISH procedures. **Results:** The BAP probe identified *JAZF1* rearrangement in 17 of 37 (46%) of sarcoma specimens. These include *JAZF1* rearrangement in 7 of 11 (64%) primary ESS, 6 of 9 (67%) metastatic ESS, 3 of 8 (38%) ESN, and 1 of 9 (11%) UES tumors. The 26 control specimens were confirmed to be normal and were used to establish false positive cutoff values for recurrent signal patterns of this probe. **Conclusions:** A lab developed FISH assay was validated to detect *JAZF1* rearrangement in a subset of ESS, ESN and UES. This FISH probe will be useful in the diagnosis of endometrial stromal sarcomas, particularly when ESS occurs as a metastatic lesion or exhibits variant morphology.

ST14. Evaluation of the OpenArray Platform for a Noninvasive Melanoma Detection Assay

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Introduction: When detected and diagnosed early, melanoma is highly curable. However, the current means for melanoma detection depends upon visual and optical cues that, although sensitive, have low specificity. Only 3% to 10% of lesions biopsied for suspicion of melanoma are found upon histopathologic evaluation to harbor the disease. In addition, 10% to 35% of such biopsies can have discordant pathology readings. We recently identified a multi-gene genomic classifier by means of epidermal genetic information retrieval (EGIR) (*Brit J Dermatol* 2001 164:797) that was found to be 100% sensitive and 88% specific for detection of both *in situ* and invasive melanoma. The microarray assays to discover this classifier used sub-nanogram amounts of RNA, non-invasively harvested by adhesive tape stripping of stratum corneum overlying a lesion deemed suspicious for melanoma. The objective of this study is to determine whether the OpenArray (OA) platform (Applied Biosystems, Inc.) has adequate sensitivity and reproducibility to enable translation of an EGIR-based melanoma detection assay into the clinic. **Methods:** Pigmented skin lesions, suspicious for melanoma, were tape stripped and then biopsied as per standard of care. Total RNA isolated from the tape by EGIR was pre-amplified by 14 cycles of PCR and then assayed on a custom OA plate, using TaqMan real-time PCR (qPCR). The custom OA plate contained 15 gene targets, based on our discovery research for a melanoma classifier, as well as 3 internal control gene targets. **Results:** Using this OA platform, we determined that as few as 100 copies of target mRNA can be detected in EGIR-harvested specimens. In addition, OA qPCR assays exhibited > 4-log linear dynamic range (LDR) and had a coefficient of variation (CV) < 3%. Some 118 EGIR specimens (57 *in situ* and invasive melanomas, 61 nevi) were analyzed and, following normalization, assessed by means of the TreeNet algorithm (Salford Systems, Inc.) for class prediction. This 15-gene classifier was found to be 100% sensitive and 85% specific with a ROC AUC > 0.912 for discrimination of *in situ* and invasive melanoma from nevi. **Conclusions:** These results demonstrate that the OA qPCR platform provides sufficient sensitivity, reproducibility, and LDR for assay of picogram quantities of RNA present in EGIR-harvested skin specimens. In addition, our initial testing of this technology shows that it is highly accurate for melanoma detection and suitable for further development and implementation in the clinical setting.

ST15. Development of a MicroRNA-Based Classification Model for Differential Diagnosis of Pancreatic Ductal Adenocarcinoma in Fine Needle Aspirates: Results from a Multi-Center Study

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Introduction: Differential diagnosis between chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) can be difficult. PDAC can arise in the background of CP and can cause secondary inflammatory changes due to obstruction of the pancreatic duct. PDAC also shares many of the microscopic and imaging features with CP. Endoscopic ultrasonography-guided fine needle aspiration (EUS-FNA), the most accurate preoperative diagnostic modality for pancreatic masses, has a reported false-negative rate of up to 30%. In this disease setting, an inaccurate diagnosis can result in a delayed surgery for a potentially curable lesion. Thus, molecular markers, such as microRNAs, may be valuable in resolving challenging, ambiguous or indeterminate cases. Herein, we will discuss three different strategies that were applied in the development of a miRNA-based classification model for the differential diagnosis of PDAC in EUS-FNAs. **Methods:** Total RNA from fresh frozen, FFPE and EUS-FNA specimens preserved in RNARetain® (Asuragen) was extracted using methods optimized for recovery of small RNAs. miRNA candidates were initially selected using commercially available high throughput miRNA expression microarray platforms (Ambion, Agilent) and their expression was verified using TaqMan miRNA Assays (Applied Biosystems). Three classification models were constructed to achieve a differential diagnosis of PDAC using EUS-FNA. Model 1 contains miR-196a and miR-217 (previously published work on high tumor content FFPE samples). Model 2 comprises of miR-196a, miR-210 and miR-375 and was developed using low tumor content fresh frozen, FFPE and FNA samples. Model 3, which consists of unique miRNAs, was developed using microdissected fresh frozen and low tumor content FFPE samples. Initial performance of the models was evaluated on different FFPE sample sets, but validated on a common set of EUS-FNAs. **Results:** In the development phase using FFPE specimens, all models tested (Model 1, 2, 3) showed promising performance with Area Under Curve (AUC) ranging from 0.96-0.99. During model migration to EUS-FNA specimens, only Model 3 retained its accuracy to distinguish PDAC from CP showing an AUC of 0.88. **Conclusions:** Although all three classification models were expected to perform differently when applied to EUS-FNA specimens as compared to FFPE specimens, it was not clear a priori which model would perform best. The results herein reinforce the importance of considering sample type and bias in discovery and development of biomarkers. Furthermore, the results indicate that miRNA-based classification models are able to aid in differentiation between PDAC and CP using EUS-FNA specimens.

ST16. Analysis of *BRAF* and *EGFR* Mutations in Thyroid Carcinomas

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Introduction: The worldwide incidence of thyroid carcinoma is steadily rising with an expected concomitant increase in disease-associated mortality. Four major subtypes of thyroid carcinoma are recognized: papillary (PTC), follicular, medullary, and anaplastic, each with a unique histologic and molecular signature. Activating mutations (T→A 1799; V600E) in the v-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) kinase

are associated with aggressive disease in PTCs. Epidermal growth factor receptor (*EGFR*) is an upstream receptor in the *BRAF* signaling pathway. In certain lung adenocarcinomas, *EGFR* exon 19 deletions (del 2235-2259/2236-2250; del E746-A750) or activating mutations in exon 21 (T→G 2573; L858R) are associated with poor clinical outcomes. Given that *BRAF* mutations are present in only a subset of thyroid carcinomas and that oncogenic *BRAF* and *EGFR* mutations are mutually exclusive, we investigated the contribution of *BRAF* and *EGFR* mutations in different subtypes of thyroid carcinoma. **Methods:** 60 cases of thyroid carcinoma from a single academic institution from 2002-2008 were tested. The histologic carcinoma subtypes tested included PTC (18), follicular (33), medullary (2), anaplastic (6), and an unclassifiable poorly-differentiated carcinoma (1). Metastatic carcinoma was present in 20 cases. A single paraffin block for each case was selected for mutational analysis. DNA was extracted using a Qiagen DNA Mini Extraction Kit. Mutational analysis was performed by pyrosequencing using a Qiagen Pyromark Q96. **Results:** DNA was successfully extracted from 59 of 60 paraffin blocks. The DNA yields ranged from 12.7 – 2661 ng/µl. One case of follicular carcinoma yielded only 4.4 ng/µl of DNA and was insufficient for analysis. 10% (6/59) of the carcinomas tested positive for the V600E *BRAF* mutation while none (0/59) of the carcinomas tested positive for either *EGFR* exon 19 deletions or exon 21 activating mutations. Of the six carcinomas that harbored the V600E *BRAF* mutation, there were four cases of metastatic PTC and two cases of anaplastic carcinoma. **Conclusions:** Our data confirm the aggressive nature of carcinomas with V600E *BRAF* mutations as 100% of cases were either metastatic PTC or anaplastic carcinoma. No E746-A750 deletions or L858R *EGFR* mutations were identified suggesting that alternate *EGFR* mutations or other oncogenic mutations are involved in the carcinogenesis of these malignancies. With the paucity of current chemotherapeutics for progressive metastatic thyroid carcinoma and the advent of selective inhibitors for cancer treatment, further investigation of molecular thyroid carcinogenesis is warranted.

ST17. Beyond V600E: Comprehensive Genotyping of *BRAF* Codon 600

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Introduction: *BRAF* mutations at codon 600 occur in 70% to 90% of metastatic melanomas and somewhat less frequently in colorectal, ovarian, thyroid and other tumors. The V600E (T1799A) activating mutation occurs with highest frequency across all tumor types, while lower frequency variants are of emerging importance. Pan-RAF and mutant-specific *BRAF* inhibitors have shown promising results in clinical trials. Lower frequency variants such as V600K (10% to 15%) have displayed differential responses to inhibitor therapies, demonstrating the importance of distinguishing between these variants. We have developed a robust semi-quantitative allele specific PCR assay that categorizes the codon 600 variants for identification of patients that may benefit from targeted therapies. To complement these AS-PCR assays, clinical samples with high mutant allele percentage (>10%) are reflexed to our validated Sanger sequencing assay. Together these assays allow complete characterization of high frequency V600E/K and the rarer variants R, M, and D, and de-novo mutations that may arise. **Methods:** Genomic DNA was extracted from immortalized human melanoma cell lines and FFPE tumor tissues using internally validated protocols. Primers were designed and optimized to distinguish mutant variants and functionally evaluated to determine specificity. Sensitivity was evaluated using serial dilutions of mutant *BRAF* into wild-type DNA. Precision and accuracy were determined using a blinded set of remnant clinical samples of known genotype. The AS-PCR assay utilizes 3 primer sets and a common TaqMan-MGB probe to generate an overlapping control amplicon and two allele-specific codon 600 amplicons that categorize E and K type variants. The Sanger sequencing assay provides bi-directional coverage spanning nucleotides 1742-1860 of exon 15 using standard PCR amplification and capillary electrophoresis. **Results:** The AS-PCR assays were designed with reference control and mutant specific amplicons of equivalent amplification efficiency. The relative sensitivity was determined to be ~0.2% mutant allele (~10 copies) in a wild-type background with absolute sensitivity approaching single copy detection by dilution to extinction of the V600E and V600K mutants. The absence of amplification from mutant specific primers on wild-type templates is required for single copy detection and achieved. Rare variants V600R, M, and D will also be detected by these assays and further characterized by the Sanger sequencing assay when present at ~ 10% to 20% mutant allele. These assays were validated using FFPE tissues from metastatic melanoma for reproducibility, precision, and accuracy with 100% concordance. **Conclusions:** The *BRAF* assays are validated for detection of common and rare codon 600 mutations in solid tumors.

ST18. Blood Cell Origin of Circulating MicroRNAs: Implications for Cancer Biomarker Studies

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Introduction: Recent studies have reported endogenous plasma miRNAs as biomarkers for solid tumors, yet little is known about the cellular origin of plasma miRNAs or mechanisms that influence circulating miRNA levels. **Methods:** We used qRT-PCR to examine expression of 85 proposed circulating miRNA biomarkers of non-hematopoietic cancers in purified sub-populations of blood cells and matched cell-free plasma from healthy individuals. To determine if blood cell counts influenced circulating miRNA biomarker levels, we correlated plasma miRNA levels to blood cell counts in a cohort of 42 consecutive residual plasma specimens collected from an academic hospital hematology laboratory and in serial plasma specimens from a patient undergoing autologous stem cell transplant. **Results:** We found that 47/85 (55%) of literature-reported solid tumor biomarkers are highly expressed in one or more blood cell type, and that 43/47 (91%) of these blood-cell expressed biomarkers were also present at considerable basal levels in healthy donor plasma (>50th percentile among all miRNAs detected), suggesting that blood cells could be a major source for these plasma miRNAs. Plasma levels of myeloid (miR-223, miR-197, miR-574-3p, let-7a) and lymphoid (miR-150) expressed biomarkers were tightly correlated with white blood cell counts, with greater than 50-fold differences between patients with the highest and lowest overall cell counts. Plasma biomarkers expressed in red blood cells (miR-486-5p, miR-451, miR-92a, miR-16) were not influenced by blood cell counts, but were significantly increased in hemolyzed specimens (20- to 30-fold plasma increase; $p<0.0000001$). Finally, in a patient undergoing autologous hematopoietic cell transplant, plasma levels of myeloid- and lymphoid-restricted miRNAs (miR-223 and miR-150) closely mirrored changes in corresponding blood counts. **Conclusions:** Blood cells are a major contributor to circulating miRNAs. Perturbations in blood cell counts can alter plasma miRNA biomarker levels by at least 50-fold, which raises concerns regarding prior studies that have attributed small miRNA differences (2 to 5-fold) to specific cancers. Future studies of circulating miRNA biomarkers for solid tumors and other non-hematopoietic diseases may be most effective if focused on miRNAs that are not highly expressed in blood cells.

ST19. Reevaluation of *HER2* Status in Breast Cancer Patients According to the 2011 Guidelines

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Introduction: *HER2* amplification is detected in about 20% of breast cancer patients. The mechanism underlying *HER2* overexpression is amplification and *HER2* overexpression effects clinical outcome, treatment and prognosis of these patients. *HER2* positive patients have poor prognosis and they are resistant to endocrine therapies mostly. The agents targeting *HER2*, like trastuzumab, are more effective in these patients. **Methods:** In our study we reevaluated formalin-fixed, paraffin-embedded (FFPE) tissue sections of 54 breast carcinoma patients admitted to our department between the years 2008 and 2011. We reviewed our FISH analysis results according to the 2011 ASCO guidelines. These patients had invasive ductal carcinoma histopathologically. All patients had 1 or 2+ IHC results and have been evaluated for estrogen and progesterone receptor status. **Results:** We detected *HER2* amplification in 22 of 54 patients. Previously, we reported ≤ 2 signal ratio as no gene amplification whereas ratios 2-4 and >4 were regarded as amplification. According to the 2011 guidelines from both ASCO and NCCN, we accepted patients with a signal ratio <1.8-2.2 as borderline. We reported these results as amplification as the algorithm in the ASCO guidelines suggests patients with ≥2 ratio are eligible for trastuzumab treatment. Four of 22 positive (18.2%) patients in the study were in the intermediate or equivocal group according to the ASCO guidelines. In addition, we suspected aneuploidy in three patients; two of them were amplification-positive and the other was amplification-negative. Our results with intermediate or equivocal FISH results seem to be similar to the literature. **Conclusions:** *HER2* status is important in targeted therapy of breast carcinoma patients as well as other cancers. Therefore, standardization of analysis and reporting parameters is important both for the patient and the clinician. We believe the new guidelines reached the aim in improving the accuracy of *HER2* amplification and it's utility as a predictive marker.

ST20. Protein Signatures for Classification and Prognosis of Gastric Cancer: A Signaling Pathway-Based Approach

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Introduction: Worldwide, gastric cancer is the fourth most common malignancy and ranked as the second leading cause of cancer death. Unlike other common cancers, the prognosis for most gastric cancer is poor and has improved little over the past several decades. Current methods have limited accuracy in predicting survival and stratifying patients with gastric cancer for appropriate treatment. We aim to identify protein signatures of gastric cancer for classification, prognostication as well as stratification for appropriate treatment. **Methods:** Protein Pathway Array (initial study) and Western Blot (confirmation) were used to assess the protein expression in a total of 199 fresh frozen gastric samples. Fifty-six paired samples were divided into a training set (n=37) and a validation set (n=19) for the identification differentially expressed proteins between tumor and normal tissues. Fifty-six tumor samples were used to identify proteins correlating with tumor and nodal staging. All of the 93 tumor samples were used to identify candidate proteins for predicting survival. An additional cohort of gastric cancer samples (n=50) was used to confirm the survival prediction of the candidate proteins. **Results:** Twenty-two proteins were differentially expressed between normal and tumor tissues. Nine proteins were selected to build the predictor to classify normal and tumor samples by cross-validation analysis. Ten proteins were differentially expressed among different T stages and 4 of these were able to separate the tumors into 2 groups (less invasive group and more aggressive one). An additional 4 proteins were associated with lymph node metastasis. Two proteins (AKT and CDK2) were identified as independent risk factors for overall survival. **Conclusions:** This study indicated that some dysregulated signaling proteins could be selected as useful biomarkers for tumor classification and predicting outcome in gastric cancer patients.

ST21. Germline Mutational Analysis of *SDHB*, *SDHC*, and *SDHD* Genes in 196 Subjects with Paragangliom/Pheochrocytoma

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Introduction: Paragangliomas are tumors arising in paraganglion tissues associated with autonomic nervous system. Pheochromocytomas and extra-adrenal paragangliomas involve the sympathetic nerves and are also called sympathetic paragangliomas. The mitochondrial SDH complex II subunit genes, *SDHB*, *SDHC* and *SDHD*, are classically considered to be involved in tumorigenicity of paragangliomas/pheochromocytomas although the mechanism is still unclear. Germline mutations in these genes result in highly increased risk for paraganglioma/pheochrocytoma. The predisposition to tumor is inherited in an autosomal dominant mode, with loss of heterozygosity in tumors. **Methods:** Peripheral blood DNA samples from 196 independent subjects affected with paragangliomas/pheochromocytomas were analyzed for the entire coding regions of the *SDHB*, *SDHC* and/or *SDHD* genes. Oligonuclceotide microarray comparative genome hybridization (aCGH) analyses were performed in 44 subjects without identifiable mutations in these 3 genes from sequence analysis. **Results:** A total of 37 different mutations/missense variants were identified in 56 unrelated families. Thus, the overall mutation detection rate is ~29% (56/196). In these 56 families, 30 had mutations/missense variants in the *SDHB* gene, 1 in the *SDHC* gene and 25 in the *SDHD* gene. Among the mutations/missense variants identified, 6 and 4 in the *SDHB* and *SDHD* gene, respectively, are novel. Intragenic deletions and duplications involving *SDHB*, *SDHC* and *SDHD* genes were not detected in all 44 samples analyzed. Interestingly, one subject affected with severe Leigh syndrome, typical of a mitochondrial disorder harbored a homozygous c.143A>T (p.D48V) missense pathogenic variant in the *SDHB* gene. Transmission of *SDHD* associated tumor was hypothesized to be paternally imprinted. However, we observed an *SDHD* case whose disease appeared to be transmitted from an asymptomatic mother. **Conclusions:** Our data demonstrated that mutations in *SDHC* is least represented among the three SDH genes. The c.380T>G (p.I127S) mutation is the most common mutation in the *SDHB* gene (4/30) and the c.242C>T (p.P81L) is the most common one (7/25) in the *SDHD* gene. Germline deletion of the SDH genes in paraganglioma/pheochrocytoma may be rare. Autosomal recessive mutations in the *SDHB* gene may cause a typical mitochondrial disorder, while heterozygosity may increase susceptibility for paragangliom/pheochrocytoma. Exceptions to paternal imprinting of the *SDHD* gene are worth investigating.

ST22. E-Cadherin (*CDH1*) Gene Mutation Could Explain at Least in Part the Pathogenesis of Korean Patients Presenting with Signet Ring Cell Gastric Cancers

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Introduction: The relationship between germline mutations in *CDH1* gene, encoding E-cadherin, and hereditary diffuse gastric cancer has been well defined in the Western population. However, the impact of *CDH1* gene mutation, despite the higher prevalence of stomach cancer, has not been well described in Asians, especially Korean population. In this study, we tested the *CDH1* gene mutation status of Korean patients with diffuse gastric cancer with familial tendency. **Methods:** We tested the mutation status of *CDH1* gene in 42 Korean gastric cancer patients with familial tendency. PCR-direct sequencing was performed for all the 16 exons and flanking regions and Multiplex Ligation-dependent Probe Amplification (MLPA) was done for the subjects with negative sequencing results using a commercial kit. The included patients all satisfied the following criteria: presence of at least two gastric cancer patients within first-degree relatives or diagnosed before the age of 40 years. For the patients with negative results for *CDH1* gene mutation and negative for P53 immunohistochemistry performed for the tumors, *TP53* gene mutation testing was done by direct sequencing method. **Results:** Two (4.8%) patients had the same known nonsense mutation (c.1003C>T, p.Arg335X) in exon 7. One was 40-year-old female and had no familial cancer history. The other was 43-year-old female and her brother and sister died due to stomach cancer at the age of 41 and 21 respectively. Although one patient had unclassified missense variant (c.715G>A, p.Gly239Arg), it was predicted as 'benign' or 'tolerate' on *in silico* prediction algorithms such as PolyPhen and SIFT. MLPA results were normal for all the patients. No patients had *TP53* mutation. **Conclusions:** Although some E-cadherin gene mutations were found in Korean patients, they cannot fully explain the possible relationship between *CDH1* mutation and hereditary diffuse gastric cancer. It implies that there might be another genetic mechanism underlying diffuse gastric cancer with familial tendency especially in Koreans. Also relatively old age of the subjects suggests that the clinical criteria for hereditary diffuse gastric cancer should not restrict the subject to younger age group only and we should pay attention to familial nature of the subjects.

ST23. Pyrosequencing Is a Highly Sensitive Method for Assessment of *MGMT* Promoter Methylation Status in Glioblastoma Multiforme in Specimens with Significant Tumor-Associated Necrosis

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Introduction: Assessment of methylation status of the O6-methylguanine methyltransferase (*MGMT*) gene promoter in high-grade astrocytomas provides valuable information including prognosis as well as prediction of response to chemotherapeutic alkylating agents such as temozolomide. Tumors with methylated *MGMT* have increased susceptibility to temozolomide and longer survival. High-grade astrocytomas often display necrosis, accompanied by variable numbers of reactive microglia. Typically, the hypercellular areas that include neoplastic cells intermixed with reactive microglia are assessed indiscriminately for determination of *MGMT* promoter methylation status. The purpose of this study is to determine the limit of a highly sensitive *MGMT* methylation assay utilizing various sized tissues with varying proportions of microglia/necrosis. **Methods:** From archival files in the department of Pathology at the University of New Mexico, 27 cases of glioblastoma multiforme, diagnosed between January 1, 2009 and December 31, 2010, were selected for this study. Representative paraffin-embedded tissue blocks containing large areas of necrosis or scant biopsies were selected for analysis of *MGMT* methylation status as well as for dual-immunohistochemical staining using glial fibrillary acidic protein (GFAP) and CD163 antibodies. DNA was extracted from paraffin-embedded tissue sections, treated with bisulfite, and subjected to PCR amplification, followed by pyrosequencing for analysis of eleven CpG sites in the *MGMT* promoter region. Methylation of eleven CpG sites was averaged and for each sample, an average greater than 5% was considered hypermethylated (based on previous validation studies). Proportions of tumor and microglia/necrosis were analyzed based on staining patterns of GFAP and CD163 respectively by Aperio® Color Deconvolution v9 algorithm. **Results:** The area of tissue analyzed ranged from 1.1 mm^2 to 260.7 mm^2 (median=78.04 mm^2), and the percent of necrosis/microglia infiltration ranged from 12% to 84% (median=42.3%) of tissue analyzed. 15 of 27 (56%) cases showed hypermethylation of the *MGMT* promoter region. The highest percentage of necrosis in a sample with hypermethylation of the *MGMT* promoter was 72%. The smallest tissue area with hypermethylation of the *MGMT* promoter (38.1%) was 4.3 mm^2, which consisted of 2.5 mm^2 of

microglia/necrosis and 1.8 mm^2 of tumor. **Conclusions:** Our study demonstrates that pyrosequencing is a highly sensitive method for reliable detection of *MGMT* promoter methylation status in biopsies with significant necrosis (up to 72%) or scant tumor tissue (down to 1.8mm^2). This robust, highly sensitive *MGMT* promoter methylation pyrosequencing assay provides valuable information to patients and clinicians even in the presence of low-level methylation, necrosis or scant specimen.

ST24. Comprehensive Approach for Molecular Testing of Thyroid FNA Samples

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Introduction: The recently revised American Thyroid Association's management guidelines recommend testing for a panel of somatic mutations (*BRAF*, *RAS*, *RET/PTC*, *PAX8/PPARg*) for nodules with indeterminate FNA cytology to help guide clinical management. We developed a comprehensive approach for testing for these mutations in thyroid FNA samples that includes a specific method for collection of thyroid cells and novel control for assessment of adequacy of FNA samples. **Methods:** Thyroid FNA samples were collected during an ultrasound guided FNA procedure; cells that remained in the FNA needle after preparation of a cytology smear and needle washings were collected into a nucleic acid preservative solution (Roche) and stored at -20°C until molecular testing was requested. Controlling adequacy of collected FNA sample: Due to common contamination of thyroid FNA with non-thyroid cells (blood cells, stromal cells, etc.), assessment of thyroid cell quantity is important prior to molecular analysis. The method we developed is based on detecting difference in expression of *GAPDH*, which is uniformly expressed in all cells types, and the cytokeratin gene *KRT7*, which is expressed only in few distinct types of epithelial cells including thyroid cells. Expression of both genes was detected by real-time RT-PCR on the ABI7500. CtKR7 - CtGAPDH <3.5 was used as a cut off. Detection of mutations: The *BRAF*, *NRAS*, *HRAS*, *KRAS*, *RET/PTC*1, *RET/PTC*3, and *PAX8/PPARg* mutations were detected by real-time PCR and RT-PCR techniques. **Results:** For the past two years, 1715 freshly collected thyroid FNAs with indeterminate cytological diagnosis or suspicious for malignancy were tested using this approach at the at the UPMC Molecular Anatomic Pathology laboratory. We found insufficient amount of nucleic acids in 3% and insufficient amount of thyroid cells in 9% of FNA samples, and these samples were rejected for mutational analysis. In the remaining samples, 181 mutations (80 *BRAF*, 56 *NRAS*, 21 *HRAS*, 2 *KRAS*, 11 *RET/PTC*1, 3 *RET/PTC*3, and 8 *PAX8/PPARg*) were identified. When corresponding surgical material was available for mutational analysis, a 95% correlation in mutational status was found between the FNA and resected specimens. **Conclusions:** We report a comprehensive approach for detection of mutations in thyroid FNA samples that allows high accuracy of molecular testing. Collection of residual thyroid FNA material directly into preservative solution provides high quality of nucleic acids and does not compromise cytological examination. The addition of *KRT7* control is useful for assessment of proportion of thyroid cells in FNA samples and helps to eliminate false negative results.

ST25. Cancer Subclones and Treatment Prediction: Mutation Detection on FFPE Tissue Sections *in Situ* by Padlock Probes and Rolling Circle Amplification

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Introduction: Activating oncogene mutations are targets for a new generation of cancer drugs. The standard assays for mutation diagnostics are based on DNA-extracts from crude tumor sections that often contain a mixture of distinct neoplastic cell populations – dysplasia, *in situ* cancer, well differentiated and poorly differentiated invasive carcinoma. However, little is known about the impact of tumor heterogeneity with regard to mutation status in different subclones during tumorigenesis, and if this is important for treatment response and prognosis. **Methods:** We have developed an RNA-based mutation assay that can target a set of oncogene mutations in a multiplex fashion *in situ* on routine tumor tissue slides by use of multiple mutation specific padlock probes and rolling-circle amplification. **Results:** The distribution of wild-type (green rolling-circle products) and mutated (red rolling-circle products) alleles were determined for single cancer cells in different parts of heterogeneous tumors without the use of microdissection. We demonstrate reliable detection of *KRAS* and *EGFR* mutations in colon and non-small cell lung cancer samples, on cytologic tumor imprints as well as on fresh-frozen and formalin-fixed, paraffin-embedded tissue sections. **Conclusions:** This *in situ* method offers single cell mutation detection for prediction of response to targeted therapy, such as *EGFR*-inhibitors, and holds great promise as a tool to investigate the role of oncogenic mutations in complex tumor tissues.

ST26. Utility of SNP Array for Distinguishing Independent Primary Tumors from Metastases

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Introduction: Cancer can present at two or more sites as independent primary tumor(s) or as metastatic disease. Distinguishing between these conditions is critical in the diagnosis, staging and management of patients. Currently, loss of heterozygosity (LOH) analysis may help in determining the clonality of these lesions but it is often limited due to small number of loci used for analysis. In this study we present the utility of single nucleotide polymorphism (SNP) microarray for distinguishing new independent primary tumors from metastases. **Methods:** Formalin-fixed, paraffin-embedded tissues from seven patients who had been previously analyzed with LOH clonality studies at our institution were retrieved. Seven cases, comprising of fifteen tumors and seven matched normal tissues were microdissected and DNA was isolated using a Qiagen kit and analyzed by the Affymetrix 250K Nsp SNP microarray. SNP array data was analyzed using CNAG v.3.2.0.0. **Results:** By SNP array, 4 of 7 patients had the secondary lesions being a metastasis from primary tumor and 3 had independent primary tumors. By LOH analysis, in 5 of 7 patients the secondary lesions were found to be metastases and 2 were independent primaries. Concordance rate was 87% and the discrepant case represented a lung adenocarcinoma presenting in three different sites with varying differentiation. SNP array showed high correlation in detection of deletions found by LOH. In addition, SNP array was able to detect additional regions of deletion, amplification, and uniparental disomy (UPD), which increased power of the analysis. **Conclusions:** Utilization of SNP microarray analysis allows for characterization and distinguishing independent primary tumor(s) from metastases. This technique has high correlation with the existing LOH analysis and provides higher confidence in interpretation by including regions of amplification and deletions in context of the whole genome. A larger study to confirm our findings as well as explore statistical methods to support the use of this technology in clinical practice is in progress.

ST27. Evaluation of the Prognostic Utility of miRNA and Commonly Proposed Genetic Markers in Stage II Colon Cancer

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Introduction: The standard form of treatment for stage IIA colon cancer is surgery alone, however, there is variable prognosis, indicating that other factors apart from clinical stage influence the outcome. Several markers have been proposed to contribute to this variation in prognosis, among which include mutational status of *KRAS* gene, microsatellite instability (MSI), expression of mismatch repair proteins and, more recently, microRNA (miRNA). To date, most of these markers have been evaluated at an individual level. Due to the complexities of tumor biology, we evaluated the multiple parameters indicated above in retrospectively collected colon cancer samples with known clinical outcome. This work extends our previous results suggesting the prognostic utility of miRNA in stage II colon cancer. **Methods:** We examined the association of mutant *KRAS*, MSI status, expression of *MSH2*, *MSH6*, *MLH1*, *PMS2* and *p27*, and miRNA in 95 FFPE tumors consisting of 50 non-recurrent and 45 recurrent colon cancer cases. The expression of 835 miRNAs was examined using the Affymetrix Gene Chip V1.0 microarray and select individual miRNAs were further examined using RT-qPCR assays. MSI status was examined using a fluorescent PCR-based assay and capillary electrophoresis for fragment size determination. Mutations in *KRAS* codons 12 and 13 were determined using direct sequencing on PCR products amplified from DNA isolated from FFPE tissues. The expression of MMR proteins MHS2, MHS6, *MLH1*, *PMS2* and *p27* were determined by IHC analysis. **Results:** *KRAS* mutations in codons 12 and 13 were detected in 26% of cases. Of the mutant *KRAS* cases, 91.6% were MSS, making mutant *KRAS* and MSI almost mutually exclusive. 41.6% of the mutant *KRAS* had no recurrence, while 58.3% had disease recurrence within 5 yr, suggesting that mutant *KRAS* is not associated with disease recurrence. Within the MSI cases, 66.7% had no disease relapse while 33.3% had relapse within 5 years. Absence of *MLH1* and *PMS2* was highly associated with MSI-H and good prognosis. The majority of the recurrence cases 36/45 (80%) had MSS, suggesting that microsatellite stability is associated with poor prognosis. We observed differential miRNA expression based on tumor location, MSI status, *KRAS* status and recurrence status **Conclusions:** Multi-marker evaluation may provide a more accurate assessment of prognosis in stage IIA colon cancer and may lead to improved disease management. We are currently performing larger validation studies in independent sample cohorts to evaluate the clinical utility of these miRNA in combination with other genetic markers indicated above.

ST28. Validation of *KIT* Assay in New Mexico Cancer Center Patients

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Introduction: *KIT* gene encodes a receptor tyrosine kinase that plays a major role in cellular proliferation and survival. Activating *KIT* gene mutations have been identified in multiple malignant neoplasms, including gastrointestinal stromal tumors (GIST), melanoma, acute myelogenous leukemia (AML) and mastocytosis. The mutational status of *KIT* has important clinical implications, including targeted therapy, prognostic indicator, and predictor of response to tyrosine kinase inhibitor therapy. In this study, we successfully identified clinically relevant *KIT* mutations in exons 9, 11, 13, and 7 using high resolution melting (HRM) curve analysis and pyrosequencing platforms in New Mexico Cancer Center patients. **Methods:** *KIT* mutations analysis was performed on DNA extracted from paraffin-embedded diagnostic tissue blocks from 81 GISTs, 70 melanomas, 10 AML with inv(16), 9 AML with t(8;21) and 16 mastocytosis, using HRM curve analysis on the Light Scanner 32 and pyrosequencing analysis on the PyroMark Q24 System. Mutations identified on screening using HRM were further characterized by direct sequencing on the ABI3130 Genetic Analyzer. Exons 9, 11, and 13 were targeted for *KIT* mutations in GIST and melanoma, using HRM. Mutations of codons 816, 820, 822, and 823 of exon 17 of *KIT* were targeted using pyrosequencing. **Results:** *KIT* mutations were identified in 56 of 81 (69%) GIST specimens including 6 in exon 9, 43 in exon 11, 2 in exon 13, and 5 in exon 17 (4 with N822Y and 1 with Y823H). Direct sequencing revealed multiple novel mutations in exon 11including 575-577del, V560-L576 del, and 566-571del in GIST cases. *KIT* mutations identified in 3 of 70 (4%) melanoma including 2 in exon 11 and 1 in exon 17 with N822Y. Exon 17 *KIT* mutations were found in 4 of 19 (21%) AML cases including D816V in 1 AML with inv(16), N822S in 3 AML with t(8;21), and D816V in 9 of 16 (56%) mastocytosis cases. **Conclusions:** Our results indicate that HRM assay for exons 9, 11, and 13 in conjunction with pyrosequencing for exon 17 of *KIT* gene accurately identify *KIT* mutations in clinically relevant neoplasms. Most mutations that were identified in our study are similar to those described previously. However, we identified several novel mutations in exon 11 that have not been described previously. Although the prevalence of mutations in GIST, AML, and mastocytosis in our study appear similar to those described previously, the incidence of *KIT* mutation in melanoma among New Mexican patients appear to be slightly lower.

ST29. Predicting Response to Combined Biologic Therapy in Metastatic Non-Small Cell Lung Cancer by Differential Expression of MicroRNAs from Serum

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Introduction: Prognosis in metastatic Non-Small Cell Lung Cancer (NSCLC) is poor with an average overall survival of only 10-12 months. Targeted biologic agents, such as bevacizumab (anti-VEGFR pathway) in combination with chemotherapy or erlotinib (anti-EGFR pathway) as a single agent, demonstrate improved survival, but their combination as first-line treatment is only now being tested. These agents are expensive and are effective in only a fraction of the patients treated, so technologies that can predict benefit are needed. MicroRNAs (miRNA) are small (~22 nucleotides) RNAs that silence genes through post-transcriptional regulation that involves either repression of translation or mRNA degradation. Although there is limited understanding about the targets of miRNAs, they have already shown utility in distinguishing different types of tissues, including non-cancer from cancer. MicroRNAs are also abundant in the blood where they circulate within microvesicles (50—90nm) called exosomes. The main objective of this study was to identify a panel of circulating miRNAs from exosomes that could be used to predict tumor response to first-line biologic therapy. **Methods:** Blood serum was obtained from four metastatic NSCLC patients enrolled into a single-arm phase II study of bevacizumab (anti-VEGFR pathway) and erlotininib (anti-EGFR pathway). Serum samples collected before and after six weeks treatment from two responding patients (tumor size decreased with treatment) and two patients who progressed (non-responders) were evaluated. Exosomes were isolated from each serum sample using ExoQuick Exosome Precipitation Solution, followed by RNA extraction using the QIAGEN miRNeasy Kit. The expression of microRNAs within each sample was then analyzed using Agilent miRNA Expression Microarray. MicroRNAs with significant changes in responders versus non-responders were identified using a pairwise analysis in GeneSifter. **Results:** We identified 37 miRNAs that displayed significant ($p<0.01$) changes in expression between responders and non-responders using pre- and post-treatment (6 weeks) samples. There were sets of miRNAs that changed significantly between pre- and post-treatment in responders compared to progressors, and miRNAs that were significantly different between responders and progressors either before or after initiation of therapy. **Conclusions:** In this pilot study, we found circulating miRNAs in the sera of metastatic NSCLC patients that appear to distinguish responders from non-responders receiving biologic therapy. Further investigation of these markers is needed to determine how early the change in biomarker profile can be detected, whether the pre-treatment signature is indicative of post-treatment changes and response, and whether these changes are due to a systemic reaction to the drugs or directly related to tumor burden.

ST30. *GNAQ* and *GNA11* Q209 Mutation Analysis in Uveal Melanoma

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Introduction: Recent studies have identified activating mutations at codon Q209 in exon 5 of either the *GNAQ* or *GNA11* gene in 83% of uveal melanoma. Both *GNAQ* (guanine nucleotide-binding protein alpha-q) and *GNA11* (alpha-11) can activate the MAP kinase pathway in melanoma. Mutations in *GNAQ*/*GNA11* provide new insights into uveal melanoma pathogenesis and are currently evaluated as predictive biomarker of response to *MEK* inhibitors in uveal melanoma. Here, we report the MSKCC experience with *GNAQ*/*GNA11* Q209 testing in metastatic melanoma using a wild-type blocking PCR assay to increase the sensitivity of direct DNA sequencing. **Methods:** To carry out this validation, we obtained 12 uveal melanoma DNA samples with known *GNAQ*/*GNA11* exon 5 mutation status. Because of the sensitivity of detecting mutations was only 50% by conventional PCR-sequencing, PCR amplification of *GNAQ*/*GNA11* exon 5 was also performed in the presence of a locked nucleic acid (LNA) oligonucleotide. This LNA was designed to suppress amplification of the wild-type *GNAQ*/*GNA11* DNA, leading to preferential amplification of the mutant allele. The PCR products of both standard and LNA-PCR were purified and sequenced. Multiple concentrations of LNA-oligonucleotide were tested to determine the concentration required for optimum assay performance. Between June 2010 and May 2011, 38 uveal melanoma (predominantly metastatic) samples were analyzed for *GNAQ*/*GNA11* Q209 mutations by direct DNA sequencing. Samples were routinely macro-dissected to enrich for tumor. **Results:** Concordant results were obtained for all validation samples. In the sensitivity study, an optimal result was obtained with a concentration of 0.4uM of LNA oligo-nucleotide, which improved the technical sensitivity of *GNAQ* and *GNA11* Q209 direct sequencing from 50% to 6.25% in both directions. Ten *GNAQ* (5 Q209P, 5 Q209L) and 17 *GNA11* Q209L mutations were identified in 22 metastatic melanomas, 4 in melanoma, and in one melanocytic tumor of the CNS (71%). **Conclusions:** The technical sensitivity of the LNA-PCR sequencing assay is well beyond that of standard PCR-sequencing. This assay was approved by the New York State Department of Health. *GNAQ*/*GNA11* Q209 mutations were found in 71% (27/38) of predominantly metastatic melanoma samples. Prospective genotyping for *GNAQ*/*GNA11* mutations in melanoma is of increasing interest in terms of selecting patients for treatment with targeted agents.

ST31. Quantitative Methylation Analysis of *H19* and *IGF2* Regions in Breast, Colon, Cervical, and Ovarian Cancer Tissues

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Introduction: *H19* and *IGF2* are reciprocally imprinted genes found on chromosome 11. Epigenetic gene deregulation is increasingly shown to play a role in many types of cancer. Imprinted genes are especially susceptible to epigenetic regulatory changes because of silencing of one allele and the dependence on proper methylation for normal function **Methods:** Quantitative Pyrosequencing methylation assays were developed for different regions of the *IGF2* and *H19* genes. Distal promoter, DMR0, DMR1, and proximal promoter regions of the *IGF2* gene and ICR1, ICR2, and promoter regions of the *H19* gene were studied on four different types of cancer tissue DNA (breast, colorectal, cervical, and ovarian). DNA was isolated from ten samples from each of these cancers along with their corresponding normal tissue. The DNA was bisulfite treated and methylation analysis was performed. **Results:** There was a significant trend that DNA isolated from the tumor tissues was hyper-methylated in non-imprinted regions of the genes, while a hypo-methylation was more commonly observed in the imprinted regions, such as the ICR region of *H19* and the DMR0 region of the *IGF2* gene. Different cancer types showed different methylation patterns. Breast cancer DNA showed a significant increase of methylation in the proximal promoter region of *IGF2*, while colorectal cancer DNA showed no change in methylation when compared to adjacent normal DNA. There are similar trends in the *H19* gene. **Conclusions:** Loss of imprinting (LOI) is an epigenetic alteration involving loss of parental origin-specific expression at normally imprinted genes. The differences in methylation patterns between DNA isolated from tumor and normal adjacent tissue across 4 different tumor types in the *H19* and *IGF2* genes indicates the critical influence of epigenetic regulation in this region on the progression of cancer. The combination of LOI and methylation changes in the promoter regions could be used as potential biomarkers for detecting breast cancer, cervical cancer, and ovarian cancer. The correlation of LOI and

methylation level change and their effect on *IGF2* and *H19* gene expression will be further investigated.

ST32. [WITHDRAWN]

ST33. Identification of Mispriming in Amplification Refractory Mutation Screening (ARMS) Used with Scorpions Technologies for the Detection of *EGFR* Mutations in Exons 18 to 21

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Introduction: *EGFR* mutations in exons 18 to 21, encoding for the tyrosine kinase domain of the receptor, have been found to be related to the response to tyrosine kinase inhibitors (TKI) in chemotherapy-refractory advanced non-small-cell lung cancer (NSCLC). *EGFR* mutations are found in approximately 10 to 19% of patients with NSCLC, and studies have shown that most *EGFR* mutants have constitutive tyrosine kinase activity and, therefore, a greater sensitivity to EGFR-targeted therapies. However, certain mutations, such as T790M, may confer resistance to TKI treatments. During molecular testing, tissue heterogeneity may mask the gene mutation status, misleading the oncologist during treatment decisions. Commercially available kits using real-time PCR aim at increasing the sensitivity of mutant DNA detection in a wild-type (WT) DNA mixture by limiting the amplification to only mutant DNA sequences. **Methods:** Here, we assessed the analytical validity of Scorpions and ARMS® technologies on DNA isolated from cell lines and FFPE sample mixtures ranging from 1% to 100% mutant DNA. These were used to establish the analytical sensitivity of the assay. In addition, WT DNA was further used to assess the mispriming phenomenon and to develop metrics to avoid false positive results. **Results:** From a total of 35 FFPE samples and 3 different cell lines we were able to determine a cutoff value for the difference between the cycle threshold (ΔCt) values obtained for mutant DNA in different proportions. Using the ΔCt metric we were able to detect *EGFR* mutations with 86.54% accuracy (PPV: 100.00% and NPV: 83.33%). This less than optimal performance was due to false positive results caused by mispriming-driven amplification. To better identify non-specific amplification, for each individual reaction, a second parameter (ΔCt2) was established from a set of negative samples. This metric alone was able to significantly ($p = 1.92E-18$) distinguish true positives from false positive results. Thus, using an algorithm that takes into account both metrics, we were able to detect as little as 1% mutant DNA with 100.00% accuracy (PPV: 100.00% and NPV: 100.00%). **Conclusions:** Scorpions and ARMS® technologies present a highly sensitive and specific method for detecting low-level mutant alleles of *EGFR* in FFPE specimens, provided that mispriming is easily identified in order to avoid false positive results. This phenomenon is enhanced when using samples with low tumor content or with limited amounts of DNA, such as those obtained from laser capture microdissection intended to enrich for tumor cells.

ST34. *KIT* and *PDGFRA* Mutation Analysis: MSKCC Clinical Testing Experience

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Introduction: Oncogenic mutations of *KIT* and PDGFR-alpha (*PDGFRA*) lead to constitutive receptor activation and downstream signaling. *KIT* mutations have been mainly detected in gastrointestinal stromal tumors (GIST) and mastocytosis, and at lower frequency in melanomas, AML, seminomas and thymomas/thymic carcinomas. Mutations in *PDGFRA* are predominantly seen in GIST. The availability of targeted therapy for the receptor tyrosine kinase mutations has led to development of molecular diagnostic tests that are being utilized in clinical practice. Here we report the MSKCC experience with *KIT* and *PDGFRA* clinical testing. **Methods:** Based on *KIT* mutation frequencies in COSMIC database, an algorithm for testing of each of the 5 type of neoplasms was developed as follows: i) GIST: exons 9,11,13,14,17; ii) Melanomas: exons 9,11,13,17,18; iii) thymomas/thymic carcinomas: exons 8,9,11,13,14,17; (iv) AML: exons 17,8,9,11,18; (v) Mastocytosis: exons 17,11. GISTs were also tested for *PDGFRA* exons 12 and 18 mutations. Between September 2009 and May 2011, 217 solid tumors (105 GISTs, 90 melanomas, 9 thymomas, 11 other tumors) and 74 hematological cases (58 AML, 9 MDS, 5 mastocytosis and 2 others) were analyzed for *KIT* and/or *PDGFRA* mutations, first by fragment analysis (*KIT* exons 9 and 11 and *PDGFRA* exon 18 for GIST only) and then, if fragment analysis was negative, by direct DNA sequencing (*KIT* exons 8,11,13,14,17,18, and *PDGFRA* exons 12,18). **Results:** We identified *KIT* and/or *PDGFRA* mutations in 90 GISTs (74 *KIT*, 16 *PDGFRA*) of which 60 were in frame deletions or insertions (indels) in *KIT* exons 9 (13 cases), 11 (42 cases) or *PDGFRA* exons 12 (1 case), 18 (4 cases), and 30 missense mutations. Nine GIST cases had second site *KIT* mutations associated with acquired resistance to Gleevec or other *KIT* inhibitors, including 4 in exon 17, 4 in exon 13, one in exon 14. Five melanomas, one thymic carcinoma, 2 AML, and one mastocytosis were positive for *KIT* mutations. **Conclusions:** *KIT* mutations were found in 70.5% (74/105) of GIST cases and *PDGFRA* mutations in 15.2% (16/105). Following our algorithm, fragment length analysis detected indels in *KIT* exons 9 and 11 and *PDGFRA* exon 18 in 56% (59/105) of GIST cases. This reduces the amount of DNA sequencing by half. *KIT* and *PDGFRA* mutation analysis is an adjunct tool in GIST diagnosis and is important in assessment of sensitivity to kinase inhibitors.

ST35. Molecular Profiling of Colorectal Cancer: Correlation with Histopathologic Findings and Microsatellite Instability Status

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Introduction: Two primary molecular pathways have been implicated in the pathogenesis of colorectal cancer (CRC). The majority are microsatellite stable (MSS) with chromosomal instability (CIN) while others display microsatellite instability (MSI) due to alterations in DNA mismatch repair genes. Mutations in various oncogenes are also common in CRC and are associated with disease progression or response to treatment. We have performed molecular profiling on a cohort of CRC specimens to detect somatic mutations frequently altered in CRC and have correlated the results with histopathological findings and MSS/MSI status. **Methods:** 100 ng of DNA representing 16 MSI-H and 35 MSS CRC samples were screened using the SNaPshot multiplex kit (Applied Biosystems). The lab developed test enables detection of 64 mutations in 8 genes and includes: *BRAF*, *KRAS*, *NRAS*, *AKT1*, *PIK3CA*, *PTEN*, *SMAD4* and *TGFBR2*. **Results:** 24/51 cases had detected mutations with 3/24 having 2 mutations identified. *KRAS* was most commonly observed (10/51) with 8/10 associated with MSS. 3/4 *BRAF* and 4/5 *SMAD4* mutations were associated with MSI-H tumors. 3/4 *NRAS* mutations were in MSS tumors. *PTEN* and *PIK3CA* mutations were exclusive to MSI-H and MSS tumors, respectively. 2/3 *PIK3CA* mutations were observed with a second driver mutation. Mutations in *AKT1* or *TGFBR2* were not detected. 12/16 MSI-H cases were located in the proximal colon while 4/16 were distributed along the transverse and distal colon. 3/16 cases were medullary carcinomas with a driver mutation detected in each and 1 with both a *BRAF* and *PTEN* mutation. 5/16 were poorly differentiated adenocarcinomas (PDA) with 4 displaying a driver mutation. The remaining 8 MSI-H tumors were moderately differentiated adenocarcinomas (MDA), with 7 having a mucinous component and 1 having no mucin but an extensive intratumoral lymphocytic infiltrate; 3 of these showed *SMAD4* mutations. 27/35 MSS cases were MDA with 5 containing a mucinous component and 10/27 demonstrating driver mutations. 8/35 MSS cases were PDA; 4/8 had histologic features typically associated with MSI with 3/4 having a driver mutation. 4/8 MSS PDA had no MSI associated histologic features and no mutations. **Conclusions:** Driver mutations were detected in 10/16 (62%) MSI-H and 16/35 (46%) MSS tumors. Most MSI-H cases (7/8) with medullary and high grade features harbored at least 1 driver mutation. In this cohort, MSS and MSI-H CRC tumors appear to display distinct molecular profiles. Studies such as these will facilitate genetically-informed treatment plans for the optimization of patient management.

ST36. New miRNA Markers in Follicular-Patterned Thyroid Tumors

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Introduction: The most difficult thyroid tumors to be diagnosed by cytology are follicular carcinomas (FC), oncocytic or Hürthle cell carcinomas (HCC) and follicular variants of papillary carcinomas (PTC,FV). Several miRNAs were previously reported as reliable markers in conventional papillary thyroid carcinoma, however, limited information is available for FC, HCC and PTC,FV. The aim of this study was to find candidate miRNA markers that can be used in diagnostically challenging thyroid FNA samples. **Methods:** Forty-three thyroid carcinomas (15 FC, 13 HCC, 15 PTC,FV), 2 benign hyperplastic nodules and 6 normal thyroid tissues were studied for expression of 380 miRNAs using Human Microarray Assays (Applied Biosystems) on ABI 7900. miRNAs were isolated using Trizol reagents from frozen thyroid tissue (n=28) and using RecoverAll Total Nucleic Acid kit (Ambion) from FFPE tissue specimens (n=23). The data analysis was performed with StatMiner v.4.2 (Integromics) and Data Assist v2.0 (Applied Biosystems) programs. Ten thyroid FNA samples were studied for expression of individual miRNAs. **Results:** miRNA expression was different between follicular variant of papillary carcinomas and follicular carcinomas demonstrating individual clusters on the unsupervised hierarchical clustering analysis. The most consistently up-regulated miRNAs in PTC,FV were miR-221, -222, -146b-3p, -146b-5p, -187, -155, and miR-375 and down-regulated miRNAs were miR-138 and miR-199a. Expression of these miRNAs was confirmed in 10 FNA samples derived from PTC,FV tumors. Both, follicular carcinomas of conventional type and oncocytic or Hürthle cell carcinomas

showed up-regulation of miR-182, -183, and -520e and down-regulation in miR-199, -411, -455. In addition, up-regulation of miR-221 and miR-222 was detected in HCCs. **Conclusions:** We identified specific up-regulated and down-regulated miRNAs in thyroid follicular carcinoma and follicular variant of papillary carcinoma, tumors that create diagnostic difficulties on preoperative evaluation of thyroid nodules. Further studies are necessary to validate their diagnostic use in thyroid fine needle aspiration samples.

ST37. Distinct Molecular Subtypes of Gastric Adenocarcinoma are Revealed by Expression Profiling of Viral and Human RNAs in Paraffin-Embedded Tissue

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Introduction: Gastric adenocarcinoma is the second leading cause of cancer death worldwide, and it is uniquely associated with *Helicobacter pylori* and Epstein-Barr virus (EBV) infections. **Methods:** To define molecular subtypes of gastric cancer, we designed an assay to measure 89 RNAs (20 viral, 69 human) chosen because they were shown in prior studies to be dysregulated in virus-associated cancer and/or in gastric cancer. The Nanostring nCounter system was used to quantify expression of these 89 RNAs as well as 4 housekeeping RNAs and 3 ERCC control RNAs that were spiked into 500ng of total nucleic acid extracted from macrodissected paraffin sections using the Roche HighPure miRNA Isolation Kit. **Results:** When applied to a series of 14 well characterized cell lines that had been paraffin embedded, the assay successfully distinguished infected from uninfected cells, and epithelial from lymphoid lineages. When applied to 102 cancers (86 gastric adenocarcinomas and 16 lymphoepithelioma-like cervical cancers) as well as 61 matched non-malignant gastric or uterine mucosa from the same surgical specimens, unsupervised hierarchical clustering revealed patterns of expression largely differentiating gastric from cervical lineages, and reactive from malignant diagnoses. None of the cervical cancers expressed EBV RNAs, in keeping with negative results by the gold standard EBER ISH histochemical assay. Interestingly, among the 86 gastric adenocarcinomas were 8 cases (9%) that expressed virtually all of the latent and lytic EBV RNAs on the panel and that also had distinct human gene expression profiles, implying that EBV is not an innocent bystander when present in gastric cancer cells. Surprisingly, 31% of reactive gastric mucosae expressed two or more EBV RNAs, suggesting that active viral infection is prevalent in stomach tissue adjacent to cancer, regardless of whether the cancer itself is infected. **Conclusions:** EBV-infected cancers represent a distinct biologic subtype of gastric adenocarcinoma. Further studies are warranted to explore the role of EBV in gastric cancer pathogenesis and tumor maintenance. This work illustrates the power of RNA expression profiling for exploring cancer biology and for molecular classification of disease.

ST38. Role of Laser Capture Microdissection and Real-Time, Allele-Specific PCR in Determining Clonality in Biphasic Tumors

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Introduction: Clonality studies using molecular techniques in solid tumors can be useful in situations where the origin of tumors or specific tumor features is in question. Some tumors including fibroadenomas and phyllodes tumors of the breast, pancreatic mucinous cystic neoplasms with ovarian type stroma, and colonic fibroblastic polyps (CFP) are consider biphasic due to the presence of both epithelial and stromal components in the tumor. It is unclear whether the stromal component is actually part of the same clone in these tumors or is a reactive response to the epithelial component. Since CFPs have been shown to harbor the *BRAF* V600E mutations, we evaluated a series of CFPs to assess the feasibility of clonality studies utilizing laser capture microdissection with a real-time, allele-specific PCR assay for this *BRAF* mutation. **Methods:** Six cases of CFPs were reviewed by examining H/E stained slides of FFPE tissue to confirm the correct histology and to select cases containing ample stromal tissue suitable for microdissection from surrounding epithelial tissue. Genomic DNA was extracted from both whole FFPE tissue sections of each polyp and stromal tissue that had been obtained by laser capture microdissection using a PixCell II system (Life Technologies). *BRAF* V600E mutation analysis was performed by real-time, allele-specific PCR for both wild-type and mutant sequences. Delta CT values (CT(wt)-CT(V600E)) were obtained for relative quantitative analysis and compared to a 1% V600E control (V600E DNA diluted in wild-type DNA). **Results:** The *BRAF* V600E mutation was present in all six (100%) of polyps tested. Laser capture microdissection yielded adequate stromal DNA for 5/6 polyps as determined by wild-type *BRAF* detection. All five *BRAF* V600E-postive polyps with successful DNA extraction from laser capture microdissected stroma showed either an absence (n=3) or a relative decrease (n=2) in *BRAF* V600E detection in the stroma. **Conclusions:** Our findings confirm the feasibility of using laser capture microdissected FFPE tissue for clonality studies. This method can be applied to biphasic tumors where the pathogenesis of the stromal component is controversial. Low-level detection of the *BRAF* mutation in two cases suggests inadvertent contamination of the microdissected stromal tissue with epithelium and further refinements in technique are likely to prevent this in the future.

ST39. Clinical Validation of *HER2* Testing in Gastric Cancer: A Pilot Study

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Introduction: In 2010, the ToGA (Trastuzamab for Gastric Cancer) trial showed that a combination of chemotherapy with trastuzumab (Herceptin) in patients with advanced stages of *HER2* positive gastric carcinoma resulted in an improvement in survival. Guidelines for scoring *HER2* gene amplification or overexpression for this tumor type have not yet been fully validated. In this study, an interlaboratory comparison of assay performance for *HER2* status by IHC and FISH in patients with both a biopsy and resection was performed. **Methods:** Formalin-fixed, paraffin-embedded gastric carcinoma (paired biopsy and resection) tissue specimens were selected after review of Surgical Pathology reports. H&E slides and blocks were retrieved from the Saint Barnabas Medical Center archives (2003 to 2011). FISH analysis was performed at two independent laboratories using the FDA-approved Abbott-Vysis PathVysion DNA probe kit. Amplification of the *HER2* gene was defined as a ratio (*HER2*:17cen) of ≥2.2 in 60 or more cells counted. IHC was performed using a monoclonal *c-erbB-2/HER2/neu* antibody from BioGenex, clone CB11 at a 1:40 dilution. Results were scored as 0-1+ (negative), 2+ (equivocal) and 3+ (positive). Scoring and interpretation of results were in accordance with the ASCO/CAP guidelines presently established for breast carcinoma. In addition, age, gender, histologic type and TNM stage were recorded. **Results:** Results from nine patients (6 females/3males - ages ranging from 56 to 95 years) are as follows: i) Nine paired biopsy/resections (18 specimens) were negative by FISH; however, heterogeneity (defined as amplification in 1/20 cells) was observed in two biopsies and one resection each from different patients. ii) Seven of nine paired biopsy/resections (14 specimens) were negative by IHC. Two remaining: one patient's biopsy and resection were both equivocal by IHC and one patient's biopsy was negative, but the resection was equivocal. iii) Correlation between FISH and IHC was 89% (8/9) in biopsies and 78% (7/9) in resections. Discrepant results were due to equivocal IHC results. **Conclusions:** We present the results of a pilot study between two collaborating institutions addressing the clinical application of *HER2* testing in gastric carcinomas (using paired biopsy/resection specimens). Correlation between IHC and FISH results was implemented to clinically validate *HER2* testing in this tumor type. This study highlights the need for further evaluation of test performance and scoring criteria for *HER2* in gastric carcinoma.

ST40. Optimization and Validation of *EGFR* Testing on FNA Cytology and Core Biopsy Samples on the Qiagen PyroMark Q24 and Rotor-Gene Instruments

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Introduction: *EGFR*-directed therapies in lung cancer patients include tyrosine-kinase inhibitors, gefitinib and erlotinib, and the monoclonal antibody cetuximab. *EGFR* mutations are important in determining selection of patients for multi-agent chemotherapy or gefitinib and testing is performed by sequencing or PCR based testing on small biopsy or fine-needle aspiration samples. Very few comparative or optimization studies have been performed on different sample types. We present an optimization study for FNA and biopsy tumor samples on the Qiagen RotorGene and the PyroMark Q24 platforms. **Methods:** 60 formalin-fixed (FFPE) biopsy, FNA and cytology tissue blocks and 35 air-dried direct smears were used in the studies. Laser capture microscopy was used to enrich for tumor in FFPE and cell block samples. Air-dried direct FNA smears were prepared and stained using the Diff-Quick (modified Giemsa protocol) method and cells identified with a marker pen. DNA was extracted from FFPE samples using the Arcturus PicoPure DNA Extraction Kit and from the Diff-Quick smears using the Zymo Research Pinpoint Slide DNA Isolation System. Quantitative measurement of mutations in codons 719, 768, 790, 858, 861 and deletions and complex mutations in exon 19 of the *EGFR* gene was performed on the Pyromark Q24 and compared with the qualitative measurement of 19 deletions in exon 19, 3 insertions in exon 20 and mutations in codons 719, 768, 790, 858 and 861 on the RotorGene instrument with the *EGFR* RGQ PCR Kit that utilizes ARMS and Scorpions Technologies. **Results:** DNA extracted from smears ranged from 0.31 ng/ μl to132 ng/ μl and from FFPE samples ranged from 5.22 ng/μl to 127 ng/ μl. All reactions from smears were valid on both the PyroMark Q24 and RotorGene while there were 2 invalids from the cell blocks and cores on the PyroMark Q24 and 4 invalids on the RotorGene. Eight positive mutations were observed from smear samples and 14 from

cell block samples. A 5% mutation cut-off limit (PyroMark Q24) was used as the limit of detection. Ten matched smear and biopsy samples showed identical results. Five samples showed discrepant results between the PyroMark Q24 and the RotorGene. **Conclusions:** Direct extraction and analysis of *EGFR* mutations from Diff-Quick smears is a convenient and robust method for FNA obtained samples. The method has been optimized for both the PyroMark Q24 and RotorGene platforms.

ST41. Evaluation of Three Different Methods for the Detection of Mutations in Exons 18 to 21 of the *EGFR* Gene

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Introduction: Somatic mutations in the Tyrosine Kinase (TK) domain of the epidermal growth factor receptor (*EGFR*) gene in lung cancers are associated with variable response to small-molecule TK inhibitors (TKIs). About 90% of mutations with favorable responses in non-small-cell lung cancer (NSCLC) occur as in-frame deletions in exon 19 or a L858R mutation in exon 21. Mutation T790M in exon 20 is the most common mutation associated with acquired resistance to TKIs. Using a sensitive assay that is reproducible and easy to interpret is key to providing accurate results for detection of *EGFR* mutations. **Methods:** DNA from 35 new and previously analyzed formalin-fixed, paraffin-embedded tissue specimens belonging to patients with NSCLC were used for *EGFR* mutation analysis by three methods; fragment analysis, pyrosequencing and Sanger sequencing. DNA from lung cancer cell lines, H1650 with an Exon 19 deletion and H1975 harboring the L858R and T790M mutations, served as positive control. *EGFR* mutations were detected using a laboratory-developed PCR with fragment analysis on the ABI 3130 genetic analyzer, pyrosequencing using the *EGFR* Pyro Kit (Qiagen), and Sanger sequencing. In the fragment analysis assay exon 19 deletions were detected by differences in the amplicon sizes and the L858R mutation was detected by an RFLP assay. Discrepancies were resolved by repeat sequencing in a different laboratory. **Results:** Out of 35 specimens tested for exon 19 deletions, 7 specimens showed deletions by all three methods. On closer analysis, 6 specimens tested by fragment analysis and pyrosequencing had a 15bp deletion and one specimen had an 18bp deletion, whereas Sanger sequencing detected five 15bp and two 18bp deletions. The L858R activating mutation in exon 21 was detected in 6 specimens using all three methods. Three specimens positive for exon 20 mutations (T790M and S768I) and one specimen identified with the G719A mutation by Sanger sequencing were confirmed by pyrosequencing. **Conclusions:** Pyrosequencing and fragment analysis are easy to perform and interpret, and showed good specificity and sensitivity for the detection of mutations in exons 18-21. Since the clinical utility of specific size deletions in exon 19 is not yet documented, the length of the deletion may not be pertinent. Performance characteristics of all three assays for the detection of *EGFR* mutations were comparable. Implementation of the assay is dependent on availability of instrumentation and experience.

ST42. Identification of CpG Promoter Methylation Gene Targets in Gastrointestinal Stromal Tumors (GISTs)

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Introduction: The role of promoter CpG methylation in GISTs is poorly understood, with few genes having been reported to be altered by CpG methylation in these tumors. We hypothesize that GISTs may be characterized by unique CpG methylation patterns and there may be increased CpG methylation of selected gene loci in higher risk GISTs. **Methods:** DNA methylation at 1,505 CpG sites in 807 cancer-related genes was evaluated using Illumina GoldenGate methylation arrays. Forty gastric GISTs (17 >5cm and 23 <5cm) were studied. DNA was extracted from paraffin sections of tumor areas. Bisulfite modified DNA was used for quantitative SYBR green methylation specific PCR (QMSP) and array studies. Methylation levels of the promoter regions of *DHCR24* (previously shown to be altered in human cancers) and *PTCH2* (hedgehog signaling pathway) genes were determined in 38 tumors by QMSP. Methylation levels of each CpG locus were measured and reported as a% of the reference actin gene. **Results:** Methylation array data showed that methylation of CpG regions represented on the array is frequent in GISTs. The methylation values (beta) in the array range from 0 to 1. The mean methylation values for all targets of GISTs < 5cm and >5cm, excluding background methylation values <.1 were 0.29 (+0.18) and 0.34 (+0.2), P<.001, respectively. Methylation values >.2 were seen in 67% of >5cm GISTs and 54% of <5 cm GISTS. To identify individual genes that may be useful to differentiate between high-grade vs. low-grade GISTs, genes with significantly higher methylation in >5cm and >5 mitoses/50 HPF GISTs were identified. Two selected genes (*DHCR24* and *PTCH2*) showed increased methylation in high-grade (>5cm) GISTs (17 cases) vs. low-grade (<5cm) GISTs (21 cases), by QMSP. Although the difference was not statistically significant (P>.05), the mean% methylation levels of *DHCR24* were 7.9 (+24.3) and 2.6 (+5.5), and mean% methylation levels of *PTCH* were 1.9 (+3.5) and 0.1 (+0.3) for high-grade vs. low-grade GISTs, respectively. There was a positive correlation between methylation levels detected by the array and by QMSP for *DHCR24* (P=3.75E-10) and *PTCH* (P=.036). **Conclusions:** This study shows frequent methylation of GISTs at CpG targets among 807 genes represented in the Golden Gate methylation array. Frequency and levels of methylation of two selected genes correlated with the array data. Our studies provide support for a role of CpG methylation in the molecular mechanisms underlying GISTs.

ST43. Evaluation of Molecular Prognosticators in a Subset of Gliomas

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Introduction: Studies indicate that *IDH* mutations are early events in gliomagenesis and that mutated *IDH1* and *IDH2* are involved as oncogenes in the progression to Grade II and III gliomas. Molecular markers such as 1p/19q co-deletion in oligodendrogliomas, *MGMT* promoter hypermethylation, 10q (*PTEN*) loss in glioblastomas, and mutations in *IDH1* and *IDH2* in diffuse gliomas are frequently used for diagnosis, subclassification and prognosis of gliomas. Recent studies have suggested that assessment of 1p/19q codeletions in combination with *IDH* mutations in anaplastic oligodendroglial tumors offer additional prognostic information. We have evaluated the presence of these markers together with *MGMT* hypermethylation in our patient specimens. **Methods:** A total of 38 formalin-fixed, paraffin-embedded gliomas were used for analysis. Twenty-six specimens from patients with an initial histopathological diagnosis of oligodendroglioma or mixed oligoastrocytoma, 4 with astrocytoma and 8 with GBM or other glial tumors were assessed for allelic status of chromosomes 1p, 19q and 10q by capillary electrophoresis Mutational status of *IDH1* and *IDH2* was assessed using real-time PCR and melt curve analysis. The presence of mutations was confirmed by sequence analysis on the ABI 3130. *MGMT* promoter methylation was assessed by pyrosequencing. **Results:** Eighteen of the 26 (69%) oligodendrogliomas and mixed oligoastrocytomas showed 1p/19q co-deletions. One astrocytoma, 1 GBM, and a third classified as a GBM with oligodendroglial features also had loss of heterozygosity (LOH) at 1p/19q. In comparison only 3/12 (25%) diagnosed as astrocytomas or GBMs had evidence of 1p/19q LOH. Of the 38 specimens analyzed, only 1 showed LOH at 10q. This astrocytoma was also observed to have the 1p/19q co-deletion. 25/ 37 (68%) specimens analyzed were observed to have the *IDH1* mutation, and 18/25(72%) of these specimens were oligodendrogliomas/mixed oligoastrocytomas. Only 1 specimen (astrocytoma) that was *IDH1* wild type was found to have the *IDH2* mutation. Hypermethylation was present in 52% of the specimens analyzed and 50% of these were oligodendrogliomas with the 1p/19q deletion. **Conclusions:** Analyzed collectively, 69% of the oligodendrogliomas and mixed oligoastrocytomas had the1p/19q co-deletion; 73% of these identified gliomas were observed to harbor the *IDH* mutation indicating that there is a significant correlation between the two prognosticators. The results further indicate that methylation status may not be a favorable indicator in the classification of tumors with oligodendroglial features.

ST44. Evaluation and Optimizing Test Sensitivity in *BRAF* Mutation Analysis

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Introduction: *BRAF* V600E encodes a *RAS*-regulated Kinase that mediates cell growth and malignant transformation kinase pathway activation. *BRAF* V600E is the predominantly occurring mutation in colorectal cancer, melanomas and thyroid cancer. More than 30 different *BRAF* mutations have been identified. *BRAF* V600E constitutes about 90% of all activating *BRAF* mutations and is the mutation commonly tested for in clinical laboratories. Because of the limited amount of tumor tissue available for testing (FFPE is the sample of choice), sensitivity and accuracy are central to this test. The aim of this study was to evaluate and implement the most sensitive and reliable method for routine *BRAF* laboratory testing. **Methods:** In this evaluation, we use three different technologies: i) real-time PCR with high resolution melting (HRM) curve analysis, ii) real-time allele-specific PCR with ΔΔCp mutant vs wild type analysis (both methods are SYBER-GREEN based) and iii) pyrosequencing (Qiagen) to analyze *BRAF* mutations from dilution of cell line and 38 patient specimens positive for *BRAF* V600E mutation. **Results:** We found that real-time PCR with HRM (2%) and real-time allele-specific PCR (2%) are more analytically sensitive than pyrosequencing (3%). When we tested 38 clinical samples all three methods have comparable clinical sensitivity (5%). Successful detection of *BRAF* mutation is dependent upon quality and percent tumor% present on the FFPE slide. For the purpose of the study, our cut-off was determined to be 30% of the tumor cell in a background of normal cells. **Conclusions:** This study suggests that all three test methods have comparable analytical sensitivity, down to 2% (real-time

PCR), which is in agreement with other publications, (3) and 3% (pyrosequencing). Clinical sensitivity is greatly dependent on the tumor cell content in the background of normal cells. In all three methods, tumor cell content <20% to 30%, increases the risk of false negative interpretation. Although none were seen here, the two real-time PCR methods may, theoretically, produce false positive results due to complex mutations present in V600E hot spot location. This risk is limited in case of pyrosequencing, where interpretation is based on detection of single base change and does not require additional confirmation. Based on above study ACL Molecular Pathology implemented a real-time PCR/HRM assay for initial screening (as a lower-cost option) with reflex to Pyrosequencing to confirm *BRAF* V600E mutation.

ST45. *KRAS* and Fluorescence *in Situ* Hybridization (FISH) Results in Pancreatobiliary Stricture Brushings Based on Tumor Localization

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Introduction: We recently demonstrated that *KRAS* mutations can be detected by quantitative PCR (qPCR) in pancreatobiliary stricture brushings and the combination of FISH with *KRAS* mutation analysis increases the detection rate of cholangiocarcinoma (CCA) over conventional cytology alone. The aims of the current study were to evaluate the sensitivity of *KRAS* mutation analysis for the detection of malignancy in pancreatobiliary brushings based on tumor localization (intrahepatic CCA; extrahepatic CCA or adenocarcinoma of the pancreas (AOP)), and to determine the relative sensitivity of *KRAS* and FISH testing. **Methods:** Pancreatobiliary brushing specimens (n=217) obtained during endoscopic retrograde cholangiopancreatography (ERCP) had previously been assessed for malignancy by routine cytology and FISH analysis. Residual brushing specimens from these patients were analyzed for *KRAS* mutations using the qPCR DxS *KRAS* mutation test kit. Five cases were excluded due to test failure (n=2) or non-diagnostic cytology or FISH results (n=3). Subsequent clinicopathologic follow-up of the 212 patients revealed intrahepatic CCA (n=27); perihilar extrahepatic CCA (n=54); distal extrahepatic CCA (n=11); AOP (n=17); other malignancies (n= 18); and benign strictures (n= 85). There were 101 patients (48%) with primary sclerosing cholangitis (PSC). **Results:** AOP had the highest *KRAS* mutation rate (71%), which was more sensitive than polysomy FISH (41%). Polysomy FISH alone had higher sensitivity than *KRAS* for all other malignancy types. Combined FISH and *KRAS* mutation analyses were complementary and demonstrated better sensitivity than FISH alone for intrahepatic CCA (59% vs. 44%, p=0.046), perihilar extrahepatic CCA (63% vs. 37%, p=0.0002), and AOP (82% vs. 41%, p=0.008). The combined sensitivity was not significantly greater than FISH alone for distal extrahepatic CCA (91% vs. 82%, p=0.32) or 'other' malignancies (72% vs. 67%, p=0.32). The combined specificity of *KRAS* and FISH was significantly lower than FISH alone (87% vs. 95%, p=0.008). Interestingly, both methods revealed a lower sensitivity for PSC-associated CCA than for sporadic CCA. Of the 35 patients with equivocal FISH results (i.e. trisomy 7 or tetrasomy), 13 (37%) showed *KRAS* positivity and all patients had malignancy on follow-up, as opposed to 20 cases (57%) with wild-type *KRAS* results in whom 50% of cases had malignancy. **Conclusions:** The findings of this study suggest that the combined sensitivity of *KRAS* and FISH is higher than FISH alone in ERCP pancreatobiliary brushings despite tumor sub-type. *KRAS* mutation analysis may be especially helpful for cases with equivocal FISH results, despite the limitation of decreased specificity.

ST46. Comparability of Sensitivity Study in *KRAS* Mutation Analysis

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Introduction: The presence of mutation in the *KRAS* (Kirsten ras) gene has been associated with poor response to anti-*EGFR* (epidermal growth factor receptor) therapies for metastatic colorectal cancer (mCRC), and most (>90%) mutations occur in codons 12 and 13 of exon 2 of the *KRAS* gene. The *KRAS* mutation test will become an important aspect of mCRC patient therapy management. Because of the limited amount of tumor tissue available for testing, sensitivity and accuracy are central to this test. **Methods:** In this study, we use three different technologies: i) real-time PCR with melting curve analysis, ii) modified traditional Sanger sequencing (ABI), and iii) Pyrosequencing (Qiagen). When analyzing *KRAS* mutations from three cell lines (3 known mutations) and 63 patient specimens positive for *KRAS* mutation, 10 different mutation types were seen. **Results:** Cell lines SW-480 (G12V), LoVo (G13D), A549 (G12S) were diluted with a known wild-type sample in serial dilution of 50%, 20%, 10%, 5% and 1% mutant and analyzed by all three methods. All levels were detected in 7-9 repeats down to 1% mutant by real-time PCR/MT and down to 5% by sequencing assays. A total of 63 *KRAS* samples (predominantly colon, liver and lung FFPE tissues) were run by all three methods. Out of the 63 positive samples all were detected. Tested samples contained percentage range of tumor cells (75-<30). Additionally 10 samples with tumor cell 30% were run in repetition all of them were detected, therefore we determined our cut-off to be around 30% tumor cells. All of the 63 samples showed the same *KRAS* type of mutation by all three methods. **Conclusions:** This study suggests that all three test methods have comparable analytical sensitivity (LOD): down to 1% for real-time PCR/MT and 5% for both modified Sanger and pyrosequencing. Although clinical sensitivity of all three methods is 100%, we chose to utilize PCR/MT as a screening method to accurately and more economically identify the negative cases requiring no further analysis. Positive cases are then reflexed to pyrosequencing for confirmation and proper identification of individual nucleotide mutation. In this process, the pathologist plays a crucial role in selecting appropriate tumor specimens for testing and result confirmation.

ST47. Comprehensive Next Generation Sequencing from Formalin-Fixed Tissue Representing a Range of Specimen Ages and Tissue Types

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Introduction: Comprehensive cancer genomic testing using Next-Generation Sequencing (NGS) technology identifies many more mutations than traditional methods. Some of these mutations, whether previously known or novel, may aid therapeutic decision making. To realize its full potential to help inform clinical cancer treatment, an NGS-based test must perform well on clinically relevant tissue specimens, principally formalin-fixed, paraffin-embedded (FFPE) surgical resections, needle biopsies, or fine needle aspirates that may vary significantly in age. Furthermore, detection of somatic mutations in impure and heterogeneous tumor specimens requires high depth of coverage, in contrast to that of germline variants. This is challenging in that formalin fixation degrades nucleic acids, making molecular analyses challenging for such samples. Here we present a targeted NGS-based test that delivers high quality sequencing results from FFPE specimens representing four tissues of origin and ranging from one to eleven years old. **Methods:** DNA was isolated from three 20µm sections each from 54 FFPE specimens (9 breast, 14 colon, 18 lung, and 13 renal tissues) ranging from one to eleven years old. Indexed, ligation-based sequencing libraries were constructed using 200ng of DNA from each specimen. Solution-based hybrid capture was used to enrich for ~200 cancer-related genes and the selected libraries were paired-end sequenced on an Illumina HiSeqTM 2000 platform. **Results:** All 54 samples produced successful (>500ng) libraries, with 52/54 yielding more than 2µg, and were then successfully hybrid captured and sequenced. After removing PCR duplicates, the median coverage across all 54 samples averaged 1,000 with a range of 390 – 1,500. The library yields and median coverage were similar across all specimen ages and tissue types. This high depth of coverage allows for high sensitivity to detect mutations present at frequencies as low as 5%. Mutations consistent with expectations for each tissue type were observed, including mutations in *APC*, *PIK3CA*, *KRAS*, and *TP53*. Additionally, error rates for the FFPE samples were comparable to those of cell-line sample controls from the same sequencing run. **Conclusions:** Our study demonstrates that high quality NGS data routinely can be obtained from the small amounts of tumor present in FFPE cancer tissue specimens representing a wide range of sample ages and tissue types. We believe these results indicate that comprehensive NGS-based testing should emerge as a routine part of cancer clinical trials and patient care.

ST48. *EGFR* Mutation Analysis in Non-Small Cell Lung Cancers (NSCLC): Experience from a Southern Taiwan Molecular Diagnosis Core Center

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Introduction: Epidermal growth factor receptor (*EGFR*) mutation analysis detects *EGFR* gene mutations in tumor specimens of patients with non-small cell lung cancer (NSCLC). *EGFR*, when activated, plays a role in cellular tumor growth and proliferation and is the target of tyrosine kinase inhibitors (TKI). Recent studies have indicated that *EGFR* mutations are associated with clinical response to gefitinib. Interestingly, all of these mutations occur within *EGFR* kinase domain. These mutations resulted in increased tyrosine kinase activity and were sensitive to gefitinib treatment. **Methods:** Paraffin-embedded tissues and needle biopsies of lung specimens (255 total) were collected from Cheng-Kung University Hospital between September 2009 and March 2011. The TK domain of *EGFR* coding sequence, from exons 18 to 21, was amplified by PCR and nested PCR conditions. PCR products were purified by QIAmp Tissue FFPE purification kit (Qiagen) and sense and antisense sequences were obtained by using forward and reverse internal primers, respectively. Each exon was sequenced using the BigDye Terminator Cycle sequence following the PE Applied Biosystem strategy and Applied Biosystems ABI PRISM 3500 DNA Sequencer (Applied

Biosystem, Forster City, CA). All mutations were confirmed by performing two independent PCR amplifications. **Results:** The 255 samples include 160 adenocarcinomas, 23 of non–small cell lung cancers/carcinoma 4 squamous cell carcinomas (SCC), and 57 cases whose pathology reports are not available. Among 255 samples, 53% (142/255) of the samples were found to harbor mutations. Most mutations were detected in exons 19 (45.7%) and 21 (45.7%). The mutation rates in adenocarcinoma, NSCLC/carcinoma, SCC and cases with no pathology report were 57% (91/160), 35% (8 /23), 0% and 49% (28/57), respectively. For the 17 patients treated with gefitinib, 10 of the 11 responders had *EGFR* mutation; the remaining six responders have no mutations. The mutations are rather complex in that altogether 23 different mutations were observed, and 8 tumors carried 2 mutations. The *EGFR* mutation rate in patients was associated with female than in man (61% vs.39%; p=0.004), never smoking history than smoking (79% vs. 20%; p=0.023), but not associated with age or cancer stage. **Conclusions:** The *EGFR* mutation rate detected in this study is 53%. The high mutation rate found in this study is in agreement with previous studies that *EGFR* mutations were more prevalent in Asian subjects with adenocarcinoma, are associated with non-smoking history and female than their counterparts.

ST49. Activating *EGFR* Mutations among Patients with Non-Small Cell Lung Cancer from India

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Introduction: Tyrosine kinase inhibitors (TKIs), targeting the epidermal growth factor receptor (*EGFR*), have replaced chemotherapy as the first line treatment of choice for advanced non-small cell lung cancer (NSCLC). However, not all patients respond to therapy. In this context, somatic mutations in the *EGFR* gene (exons 18-21) have proven to be reliable predictors of response to therapy. But there is limited data on *EGFR* mutations among patients with NSCLC from India. In this study, we analyzed the frequency of these mutations by PCR-sequencing and also attempted to determine the utility of bi-directional PCR amplification of specific alleles (Bi-PASA) in detecting *EGFR* mutations. **Methods:** DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues of 46 cases of NSCLC obtained during the period January 2008 to March 2011, after manually microdissecting from areas that had ~40% tumor. The DNA was amplified targeting exons 18-21 of the *EGFR* gene and the products were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit. Bi-PASA was performed on a subset of 20 samples using previously published allele specific PCR primers targeting exons 19 and 21, including DNA from NCI-H-1650 and NCI-H-3255 as controls respectively. **Results:** Of the 46 cases included, 43% were positive for mutations, with deletion in exon 19 (del E746-A750) being most frequent (70%). Mutations were also seen in exons 21 (L858R), exon 20 (mutation H773 ins NPH) and at exon 19 (del L747-P753 ins A and del L747-P753 ins S). Fifty-four percent of the women (n=13) included in the study had mutations while only 39% of men (n=33) carried mutations. The Bi-PASA assay was performed on a subset of 20 samples and primers AQ, which amplify the wild-type sequence of *EGFR* 19 was positive only in 15/20 samples. Primers PB that are designed to detect nucleotide deletions at E746-A750, amplified only 4/5 samples with this deletion. Similarly the G reaction specific for L858R mutation in exon 21 was positive only in 2/3 cases with this mutation. **Conclusions:** The frequency of *EGFR* somatic mutations among patients with NSCLC in our study population is higher than the incidence in the western world but similar to the reports from the Asian region. Allele specific PCR, though easy to perform and useful in resource limited settings, will have to be assessed on a larger data set to determine its true utility.

ST50. *EGFR* Gene Amplification and *MGMT* Gene Promoter Methylation in Gliosarcomas: Series of 31 Cases with Clincopathological Features

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Introduction: Gliosarcomas (GS) are rare WHO grade IV primary biphasic brain tumors composed of high grade glial and mesenchymal components with either similar or worse prognosis than glioblastomas. **Methods:** Archives of the departmental files of the last nine years were reviewed and cases of gliosarcoma, where paraffin blocks and reasonable clinical information were available were included for the study. All these cases were evaluated for *EGFR* gene amplification by fluorescence *in situ* hybridization and *MGMT* gene promoter methylation by gel-based methylation PCR assay, in addition to routine histomorphological and immunohistochemical evaluation. **Results:** Total 31 cases with age range of 12-70 yrs (median: 50yrs and mean: 47.4 yrs) and sex ratio: 6.75:1(M- 27; F-4) were noted. Commoner sites were temporal (n-12) and frontal (n-10); while other sites were parietal (n-4), occipital (n-2), intraventricular, cerebellopontine angle and pineal (one case each). One case each occurred in treated patients of medulloblastoma and adenoid cystic carcinoma of lip. One case showed an extracranial direct contagious extension into the temporal fossa and one showed systemic metastasis to iliac bone. Histologically, all except for one case showed an intricate admixture of glial and mesenchymal components. One case (known treated case of adenoid cystic carcinoma) showed a predominantly sarcomatous component with very scant (<5%) glial component. Myogenic differentiation was noted in six cases, one case showed chondrosarcomatous differentiation and in rest of the cases no specific differentiation of the sarcomatous component identified. Additionally, three cases showed admixed population of small undifferentiated small cell component. TP53 immunopositivity was noted in 27 cases and negative in 4 cases. MIB-1 LI was 15% to 40% (10% to 15% - 17, 15% to 20% -8, 20% to 30% -3 and 30% to 40% - 3). *EGFR* gene evaluation could be interpreted in 26 cases, of which one case showed amplification; while the rest of the 25 cases were non-amplified. *MGMT* gene promoter methylation results were available in two cases and both were non-methylated. The remainder are being evaluated. **Conclusions:** *EGFR* gene amplification is an uncommon molecular abnormality in GS. In addition, this series also documents the occurrence of these tumors at rare sites (intraventricular and CP angle), uncommonly as second primary tumors, heterogeneity of dedifferentiation (undifferentiated, myogenic and rarely matrix producing chondrosarcomatous) and their potential for extracranial metastases.

ST51. Sensitive Quantitative Methylation Analyses for Risk Assessment of Coexisting Dysplasia in Ulcerative Colitis Patients

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Introduction: Patients with ulcerative colitis (UC) may develop colon cancer after longstanding chronic colitis, followed by dysplasia and ultimately cancer. Dysplasia and cancer in UC develop through complex pathways, including epigenetic deregulation associated with CpG methylation of cancer related genes. The field defect in background colitis includes epigenetic changes that predispose to dysplasia and cancer development. In this study we aimed to determine whether background colitis of patients with co-existing dysplasia shows higher levels of methylation in cancer related genes *MGMT*, *MLH1*, *P16* and *TGFBR3*, as compared to colitis of patients without dysplasia. **Methods:** CpG methylation levels of the promoter regions of *MLH1*, *MGMT*, *P16* and *TGBR3* were determined in the mucosa of Colectomy specimens from 33 UC patients (12 without dysplasia or cancer and 21 with co-existing low-grade dysplasia). DNA was extracted from paraffin sections from selected areas of colitic mucosa or low-grade dysplasia. Bisulfite modified DNA was used for quantitative SYBR green methylation specific PCR (QMSP). Methylation levels of each CpG locus were measured and reported as a% of the reference actin gene. **Results:** CpG methylation of the four genes was negligible in colitic mucosa of UC patients without co-existing dysplasia or cancer, averaging 0.0 to 0.5% methylation, while CpG methylation of the four genes was significantly increased in dysplasia and associated background colitis of patients with co-existing dysplasia (mean: 4.6; 2.2; 5.1; and 1.6 in colitis and 11.2; 8.8; 4.1; and 1.4% methylation in dysplasia, for *MGMT*, *MLH1*, *P16* and *TGFBR3*, respectively, P<.001). In contrast, the methylation levels were not significantly different between matched background colitis and dysplastic lesions of UC patients with co-existing dysplasia (P>.05 for each of the four genes). Multiple logistic regression analysis of methylation levels of the four genes in background mucosa was used to derive a probability for the presence of dysplasia with a sensitivity of 100% and a specificity of 86%. ROC analysis indicated that the area under the curve for the logistic regression-derived combined probability was 0.98, as compared to 0.91, 0.86, 0.83, and 0.74 for individual *P16*, *MLH1*, *MGMT*, and *TGFBR3* levels. **Conclusions:** Sensitive quantitative methylation testing of a limited gene panel using mucosal biopsies may be a specific and sensitive test for the presence of dysplasia in patients with ulcerative colitis. Methylation test results may be used to refine the selection criteria for colonoscopic surveillance in ulcerative colitis patients.

ST52. Rapid Identification of *EGFR* Pathway Somatic Mutations in Melanoma of Varying Subtypes

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Introduction: Multiple genes associated with signaling pathways, specifically the EGFR pathway, have become targets of interest in molecular therapies for treatment of melanoma. Somatic mutations in the EGFR pathway, particularly in the *BRAF* and *NRAS* genes, are frequently seen in primary and metastatic melanomas. Rapid

identification of a large number of somatic mutations in melanoma will likely become increasingly important due to the molecular heterogeneity of mutations in primary and metastatic melanomas. In this study, we evaluated melanomas of varying subtypes with a method for rapid identification of a large number of somatic mutations in the EGFR pathway. **Methods:** DNA from eleven primary and metastatic melanomas of varying subtypes was macrodissected and DNA was extracted using the Qiagen Gentra Puregene Blood Kit. Following DNA extraction, qPCR of each melanoma was performed using the Qiagen qBiomarker™ Somatic Mutation PCR Array, directed at the Human EGFR pathway, and run on the AB 7500 Fast Real-time PCR System. The average Ct method was used to determine the mutational status of each melanoma for 85 somatic mutations across 9 genes. **Results:** Eleven melanomas were examined for 85 somatic mutations within the human EGFR pathway. Two melanomas, both metastatic, were shown to have a single mutation in the *NRAS* gene (Q61R and Q61L). The remaining nine melanomas of varying subtypes (superficial spreading, nodular, acral lentiginous, spindle cell melanoma and additional metastatic melanomas) were shown to be wild type for genes known to harbor somatic mutations in the EGFR pathway. **Conclusions:** Somatic mutations in melanomas have molecular heterogeneity that can be time consuming and costly to evaluate and identify. Rapid identification of multiple mutations of interest are more efficient and cost-effective than traditional SNP genotyping techniques. As somatic mutations are increasingly targeted by molecular therapies for melanoma, the utility of such methods are becoming an optimal tool in research, and potentially in clinical settings. In addition, further evaluation of varying melanoma subtypes using rapid identification of a comprehensive number of mutations may reveal molecular signatures specific for most commonly seen melanomas.

ST53. *MGMT* Promoter Methylation in Colorectal Cancer Is Associated with G>A *KRAS* Mutations and High Stage at Presentation

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Introduction: Promoter methylation of O6-methylguanine-DNA methyltransferase (*MGMT*), a DNA repair enzyme, is seen as an early event in the progression of various types of cancer. In Glioblastoma, *MGMT* methylation predicts a positive response to alkylating chemotherapy agents such as temozolimide. In colorectal cancer (CRC), data supporting the prognostic significance of *MGMT* methylation has been limited. Recent data show that patients with metastatic CRC and loss of *MGMT* expression may respond to temozolmide. The goal of our study was to determine the prevalence of *MGMT* methylation in CRC patients and to associate these findings with *KRAS* mutation status, pathologic features, and patient outcomes. **Methods:** Thirty-five patients (mean age 61.7; M:F 18:17; average follow up 43 mos.) with a history of CRC were included. Tumor DNA previously extracted from FFPE for determination of *KRAS* status was used for analysis. The EpiTect Bisulfite kit (Qiagen) was used for bisulfite conversion. Converted DNA was then subjected to a real-time, methylation-specific PCR to detect *MGMT* promoter methylation and ACTB as an internal control. Four patients had ACTB Ct levels >35 and were excluded from analysis. Pathologic and clinical information were collected through chart **Results:** Ten patients (32%) were positive for *MGMT* methylation. The average age of the methylated group was 66.6 (range 50-84) vs. 56.3 (range 35-81) in the unmethylated group. 50% of the methylated group had a coexisting *KRAS* mutation and of these 80% were codon 12 or 13 G>A mutations. Only 24% of the unmethylated group had a coexisting *KRAS* mutation; 60% were G>A mutations. Patients in the methylated group were more likely to have right-sided CRC (30% vs. to 24%) and to present with stage IV disease (56% vs. 35%). All patients except 2 received an alkylating agent (oxaliplatin-based chemotherapy) during treatment; these two patients were in the methylated group, had stage IV disease, and were deceased at last follow up. Mortality in both groups was similar: methylated 60% (83% CRC-related) and unmethylated 62% (85% CRC-related). **Conclusions:** Here, *MGMT* methylation was present in 32% of CRCs, similar to previous reports. These patients were more likely to have *KRAS* codon 12 or 13 G>A mutations, older age of CRC onset, right-sided lesions, and higher pathologic stage at diagnosis than the unmethylated group. Although *MGMT* methylation did not confer a survival benefit, patients with high pathologic tumor stage and *MGMT* methylation may benefit from newer alkylating agents such as temozolomide.

ST54. DNA Extraction Method for Formalin-Fixed, Paraffin-Embedded Breast Cancer Specimens and Quality Assessment Comparison to DNA Isolated from Matched Fresh Frozen Specimens for Array CGH

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Introduction: Formalin-fixed, paraffin-embedded (FFPE) specimens are frequently the only available source of solid tumor tissue for analysis, particularly in a reference laboratory setting. Unlike fresh frozen (FF) tissue, the FFPE process induces DNA fragmentation that is not ideal for array comparative genomic hybridization (aCGH). aCGH identifies copy number changes and its application in tumors is of clinical interest to detect diagnostic and prognostic genetic markers. This study was designed to identify a method of isolating DNA from FFPE breast cancer samples that yields DNA of sufficient quality to produce concordant aCGH results to those of patient-matched FF samples. We demonstrate a statistical assessment to confirm comparable DNA quality between FFPE and FF samples. **Methods:** Following IRB approval, DNA was extracted from twenty patient-matched FFPE and FF invasive breast tumors using three QIAGEN kits. Extracted DNA was quantified on a NanoDrop and fluorometer and fragment size tested by PCR. DNA was labeled using the Genomic DNA ULS Labeling kit, hybridized to Agilent arrays with a fragmented normal blood reference sample, and analyzed using DNA Analytics. We compared patient-matched FFPE and FF samples with respect to precision of the base-2 logarithm transformed foreground intensities individually for the Cy3 and Cy5 median intensity values as well as for the LogR values. Additionally, we examined the reproducibility associated with replicate assays for a set of patient-matched FFPE and FF samples. **Results:** The QIAamp kit, with protocol modifications, yielded the highest DNA concentrations and quality. Visual inspection of the data in DNA Analytics showed consistent trace alignment of the patient-matched FFPE and FF samples. Similar distributions of foreground intensities as well as derivative log ratio spread (DLRS) values were observed between matched FFPE and FF samples. The replicate FFPE assays resulted in a Pearson correlation of 0.96, 0.96 and 0.78 for the foreground Cy3, Cy5, and LogR values, respectively. Similarly, the replicate FF assays resulted in a Pearson correlation of 0.95, 0.94, and 0.61. Bland-Altman plots indicate that the discordance in the replicate assays is intensity-dependent such that higher intensity probes are more discordant than lower intensity probes. We hypothesize that the discordance is likely due to a technology limitation as both the replicate FFPE and frozen samples showed the same trend. **Conclusions:** A modified QIAamp kit method for DNA extraction from FFPE breast cancer tissue produced comparative quality and aCGH results to patient-matched FF samples as assessed by trace alignment, foreground intensity, DLRS, and concordance of replicate assays.

ST55. Utility of Gene-Expression Profiling for Reporting Difficult-to-Diagnose Cancers

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Introduction: Studies of primary and metastatic lesions of poorly differentiated malignancies find approximately 80% of lesions are identified with routine stains and immunohistochemistry (IHC). When examining only metastatic carcinomas of unknown primary, this figure drops to 65%. Limited clinical information further decreases this value. Inexperienced pathologists will arrive at erroneous conclusions more frequently. When the primary tumor site is ambiguous among three or more possibilities, there are no standard algorithms for IHC panels. Information from these stains is often indeterminate. The appropriate use of targeted chemotherapy requires knowledge of tumor type. These expensive therapies have side-effects and are only effective for select patients. A determination of primary tumor is requisite for their use. Pathwork® Diagnostics offers a Tissue-of-Origin (TOO) microarray-based test as an adjunct to IHC to reduce ambiguity of stain interpretation and provide greater accuracy in determination of cancers of unknown origin. **Methods:** This is a retrospective study of cases referred to Genoptix® for Tissue-of-origin microarray testing from April 1, 2009 through the present. Cases included: are considered difficult-to-diagnose carcinomas, have histopathology reports, have three or more possible sites of origin. Original diagnosis, TOO results, and difference, if any, between working and final diagnoses are compared. TOO relies on mRNA expression in 15 organs. Over 2,000 mRNA sequences comprise the expression profiles. Tumors are given a similarity score, ranging from 0-100, to a particular tissue based on gene expression pattern. The scores sum to 100 across all tissue types. A high score confidently assesses origin. Scores under 20 are non-contributory. **Results:** Nineteen cases of difficult-to-diagnose carcinomas were examined for original working diagnoses, gene-expression profiling and difference in final opinion based on gene-expression results. Of the nineteen cases, 12(63%) revealed a different site of origin from the pathologist-favored site. Of these, 2 did not yield high enough similarity scores to change the position of the pathologists. In 2 cases, a clinically assumed primary lesion turned out to be metastatic. 2(10%) cases were not able to arrive at a similarity score above 20, the lowest meaningful score. The remaining 5(26%) cases had results that concurred with the original interpretation. **Conclusions:** The Pathwork® Diagnostics Tissue-of-origin test is a valuable adjunct to immunohistochemistry in determining site of origin for lesions that are poorly differentiated or show staining and morphology patterns that are inconsistent with expectation. Given the current clinical need for origin determination, the Tissue-of-Origin test can provide increased diagnostic acumen for challenging specimens.

ST56. Comprehensive Molecular Analysis of High Grade Invasive Urothelial Bladder Carcinomas by Whole Genome, Mate-Pair, and Whole Exome Sequencing

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Introduction: An estimated 386,000 new cases and 150,000 deaths from bladder cancer occur annually worldwide. There is a 14-fold variation globally and a 2- to 5-fold higher incidence in males. Primary contributors to bladder cancer incidence include smoking, occupational exposures, and chronic infections. Of patients with high grade invasive urothelial carcinoma, up to 70% develop recurrences, and stage progression and death due to disease are observed in as many as 65% of patients. We attempted a comprehensive genomic analysis of high grade urothelial carcinomas by whole genome, mate-pair, and whole exome sequencing in order to better define features that may assist in improved patient management. **Methods:** Five tumor genomes from high grade invasive urothelial carcinoma with matching normal DNA were sequenced on the Illumina HiSeq2000 with 30- to 40-fold average coverage. The same five tumors as well as two well characterized bladder cancer cell lines, one karyotypically complex and one near diploid, were analyzed by the Illumina mate-pair protocol (5Kb fragment size) to identify structural rearrangements. An additional 28 high grade urothelial carcinomas with matching normal DNA (14 node negative and 14 node positive) were analyzed by whole exome capture and sequencing using the Illumina TruSeq exome capture protocol (62Mb, covering 97.2% RefSeq coding exons). **Results:** After filtering data against the paired normal samples, deep sequencing of high grade urothelial carcinomas showed 10^3 to 10^4 somatically-acquired mutations per tumor. 10 to 10^2 variants per tumor were predicted to be potentially deleterious by Ensemble Variant Effect Predictor and further mapped into cancer-related pathways. At least 12 genes were found to be mutated in more than one tumor. Some recurrent genes were known cancer-associated genes, while others likely represent passenger mutations. The mate-pair protocol successfully identified structural rearrangements using a low-coverage, tumor only strategy as compared to deep sequencing. Whole exome analysis showed, for example, that *FGFR3, TP53*, and *PIK3CA* are commonly mutated in high grade urothelial carcinomas. **Conclusions:** Whole genome analysis provided, for the first time, a comprehensive picture of the genome complexity in high grade, invasive, urothelial carcinomas. In addition, all samples showed similar tumor-specific structural rearrangements by a low-coverage mate-pair protocol, which may provide a highly sensitive, cost-effective method for monitoring patients for recurrent disease. In whole exome analysis, some differences were observed between node negative and node positive tumors. A larger data set will be required to confirm these observations.

ST57. Evaluation of *HER2* FISH with Centromere 17 Probe Scores >2.9

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Introduction: Assessment of *HER2* (*ERBB2*) gene copy number by Fluorescence In-Situ Hybridization (FISH) is a standard method for identifying *HER2* amplification in breast carcinoma. When using a single color FISH probe to *HER2*, amplification is defined as greater than 6 copies of *HER2* per cell. When using dual-color FISH probes to *HER2* and centromere 17 (CEP17), *HER2* amplification is defined as a ratio of *HER2* to CEP17 > 2.2. This calculation is designed to prevent misclassification of chromosome 17 polysomy as positive for *HER2* amplification. Recent data suggests that not all cases with increased signal from CEP17 are due to polysomy, potentially resulting in false negative *HER2* ratio scores. We have reviewed our *HER2* FISH data to identify cases with increased CEP17 signal to determine what percentage of cases would have been interpreted as negative, equivocal, or positive had the analysis been performed using a single probe assay. **Methods:** Paraffin-embedded tissues were processed per the PathVysion (Abbott, Inc.) protocol. All samples were analyzed using the PathVysion criteria and 2007 ASCO/CAP guidelines for *HER2* testing in breast cancer. Negative (non-amplified) samples had *HER2*:CEP17 ratios of <1.8. Equivocal samples had ratios between 1.8 and 2.2, and the positive (amplified) samples had ratios of greater than 2.2. Tissue sections were also evaluated by Immunohistochemistry (IHC) for *HER2* protein expression, using a *HER2*-specific antibody (clone SP3, 1:80), EDTA antigen retrieval (manual method), peroxidase-conjugated polymer, and DAB chromogen. **Results:** We identified 38 cases with CEP17 signal > 2.9. *HER2* IHC results were available for 23 of these cases. For one case, the *HER2*/CEP17 ratio was consistent with amplification. For the remaining 37 cases, the *HER2*/CEP17 ratio was consistent with negative for amplification. In the absence of the CEP17 signal, 23/37 (62.2%) would have been scored as negative, 11/37 (29.7%) would have been scored as equivocal, and 3/37 (8.1%) would have been scored as positive for amplification. Although not statistically significant, there was a trend toward increased *HER2* IHC scoring with increased *HER2* FISH score. **Conclusions:** Our data indicates that 35% to 40% of cases with increased CEP17 signal would have been reported as equivocal or positive for *HER2* amplification if a single probe assay had been performed. These results are consistent with data reported by others. This type of discrepancy needs further investigation in order to avoid failing to treat patients who may benefit from *HER2*-targeted therapy.

ST58. Whole Exome Analysis and Tumor Evolution of a Rare Malignant Myoepithelioma Tumor of Soft Tissue

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Introduction: Malignant myoepithelioma of soft tissue is a rare tumor, and little is known about best treatment practices. We report here whole exome analysis of a local recurrence of disease in the great toe, a metastatic lesion in an inguinal lymph node, and a lesion to the left lower lung, from a patient diagnosed with malignant myoepithelioma who universally failed radiation and chemotherapy. Whole exome sequencing provides an unbiased analysis of the recurrent and metastatic tumor genomes, which may illuminate mechanisms of tumor progression as well as identify molecular features that may guide patient care. **Methods:** DNA was extracted from fresh frozen tumor tissues and whole blood from a patient with metastatic malignant myoepithelioma. Indexed DNA libraries were prepared from fragmented genomic DNA and enriched by a 10-cycle PCR. Libraries were pooled and exomic fragments were captured using the Illumina exome enrichment protocol, designed to capture 62Mb of genomic DNA (97.2% RefSeq coding exons). Samples were sequenced on an Illumina HiSeq2000 (2X100 bp, paired-end reads). FASTQ files were aligned to the reference genome, filtered to identify somatically-acquired mutations, and annotated. A selection of 100 SNVs was validated by Sanger sequencing and included both a random selection of SNVs (50) and a selection of unique and common mutations between the three different tumor samples (50). **Results:** All four exomes were sequenced to an average depth of 100X coverage, enhancing the detection of low frequency variants. Most variants were common between the three tumor exomes, while a smaller number were unique. Validation studies showed high concordance with conventional Sanger sequencing, especially among those variants identified in two or three tumor specimens. Unique variants showed a higher false positive rate. **Conclusions:** Whole exome analysis has become a manageable approach to understanding tumor biology and tumor evolution in rare cancer subtypes, for which little information is available. Advantages include a comprehensive analysis of SNV and copy number alterations for protein coding regions. This approach does not currently identify structural rearrangements, mitochondrial variants, or regulatory regions, which may be important in tumor evolution and stage progression. As costs continue to decline and data analysis becomes standardized, whole exome sequencing may be a sensitive and cost-effective approach to the analysis of rare tumor types. Molecular profiling will potentially provide critical information to effectively manage patient care.

ST59. *PTEN*, *KRAS*, and *PIK3CA* Sequencing of Endometrioid and Non-Endometrioid Type Endometrial Cancers

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Introduction: Endometrial cancer is associated with numerous gene alterations that activate oncogenes and inactivate tumor suppressor genes. Many studies have assessed endometrial cancer associated genes individually on endometrioid type endometrial cancer (ETC) or non-endometrioid type endometrial cancer (NETC), so it is difficult to discern whether these gene alterations are overlapping or mutually exclusive among the different histological subtypes. The aim of this study was to characterize a set of ETC and NETC for *PTEN*, *KRAS*, and *PIK3CA* alterations by sequencing and to assess for correlations between these common alterations. **Methods:** Genomic DNA was extracted from fresh-frozen tumor samples from 86 endometrial cancers including 55 ETC (32 grade I, 12 grade II, and 11 grade III) and 31 NETC (17 carcinosarcoma, 9 serous carcinoma, 2 clear cell carcinoma, and 3 mixed tumors). Sequencing was performed for the detection of alterations in *PTEN* (exons 1-9), *PIK3CA* (exons 9 and 20), and *KRAS* (exons 2 and 3) genes. **Results:** At least one *PTEN* alteration (pathogenic mutation or variant of unknown significance) was identified in 48 (56%) specimens, including 41 (73%) ETC and 7 (23%) NETC. A *PIK3CA* alteration was observed in 16 (19%) specimens, including 8 (15%) ETC and 8 (26%) NETC. Interestingly, 56% of serous cancers had a detectable *PIK3CA* mutation, compared to only 14% of non-serous tumors. Twelve of 86 cases (14%) analyzed for *KRAS* mutations were found to have a mutation, including 8 (15%) EC and 4 (13%) NETC.

Eleven (23%) and 6 (13%) of the 48 specimens with a *PTEN* alteration also had a *PIK3CA* and *KRAS* mutation, respectively. This suggests that the co-existence of either a *PIK3CA* or a *KRAS* mutation with a *PTEN* mutation is not uncommon. None of the 16 specimens with a *PIK3CA* alteration had a *KRAS* mutation, suggesting that these alterations are mutually exclusive. **Conclusions:** Our study has assessed a large cohort of diverse endometrial carcinomas for alterations in the *PTEN*, *KRAS*, and *PIK3CA* genes. The findings of this study suggest that endometrial cancers encompass a multitude of unique molecular profiles, and larger multi-gene testing studies are needed to better understand endometrial cancer pathogenesis.

ST60. Identification of Rare Mutations in Cancers While Screening for High Frequency Mutations Using Sequenom® MassARRAY System

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Introduction: One of the important goals of personalized cancer therapy is to determine the mutational status of all therapeutically and prognostically relevant genes in the tumor. Routine screening for multiple mutations is hindered by the need for multiple platforms and limited sample. Consequently, routine screening focuses on the common mutations with the rarer, albeit clinically significant, mutations being neglected. We have employed a Sequenom® MassARRAY-based multiplex mutation detection panel as a high-throughput method to simultaneously screen for both high frequency and low frequency mutations in clinically important genes. We report the low frequency mutations identified so far. **Methods:** Following micro dissection, genomic DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tumor from several cancer types (N=425). A panel was developed to screen for mutations in 13 genes including rare mutations in four genes (*AKT1*, *PIK3CA*, *GNAS* and *GNAQ*) in addition to frequently mutated genes such as *KRAS* and *BRAF*. Briefly, 10ng DNA was subjected to PCR and single-base-primer extension using iPLEX Gold kit and samples were subjected to MALDI-TOF mass spectrometry (Sequenom®, CA). **Results:** The tumor-type specific reported frequencies of mutations shown below in parenthesis were obtained from Catalog of Somatic Mutations in Cancer (COSMIC) database. In our study, in colon adenocarcinoma, five mutations were detected in *PIK3CA* (codon (reported number of cases in COSMIC)): p.C420R (8), p.N345K (2), p.E110K (0), p.H1047R (139) and p.N88Q (7). One mutation was detected each in *AKT1* p.E17K (0), *GNAQ* p.Q209P (0) and *GNAS* p.R201C (0). In papillary thyroid cancer and breast carcinoma a single mutation p.N345K (0 for thyroid and 23 for breast)) was detected in *PIK3CA* with no mutations detected in other genes tested. In leiomyosarcoma and melanoma, a single mutation was detected in *GNAQ* p.Q209P (0 for both)). In thyroid carcinoma one mutation p.R201C (9) was detected in *GNAS* with no mutation detected in the other three genes tested. Overall, of 425 cases tested, only 19 cases were positive to at least one of the 12 mutations tested reflecting their rarity of occurrence. **Conclusions:** Sequenom® MassARRAY system can simultaneously detect frequent and rare mutations using limited sample. This approach can be applied as a convenient high-throughput technique to screen for rare mutations and obtain more comprehensive mutational profile using limited sample.

ST61. Next Generation Sequencing from Formalin-Fixed, Paraffin-Embedded Tissue

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Introduction: Formalin fixation and paraffin embedding (FFPE) is the standard for processing tumor tissue in pathology departments. Archived tissue blocks provide an important resource for molecular oncology studies, as tissue in this form and some degree of clinical follow-up is available from nearly all tumors. However, the fixation procedures that are necessary for tissue preservation compromise the quality of the DNA, making FFPE a challenging sample for many molecular applications. Clinical evaluation of FFPE tissue for oncogene mutations, such as those in *KRAS*, has been achieved, but this typically involves evaluation of a small number of nucleotides. Evaluation of an entire genome from FFPE tissue, as with next generation sequencing (NGS), is more challenging especially as compared to NGS on higher quality DNA, such as from peripheral blood leukocytes. We developed a protocol for NGS on routinely processed FFPE. **Methods:** We developed a protocol to prepare an NGS sequencing library from FFPE tissue from a melanoma sample. The sample was microdissected to enrich for tumor cells from a 5 µm slice of tissue. DNA was extracted using the Qiagen DNA extraction kit with an extended (4 days) Proteinase K incubation. DNA was then sheared to approximately 800 base pairs with the Covaris adapted, focused, acoustic technology. The SPRI-TE automated nucleic acid extractor was used for Illumina adaptor ligation. PCR was done across the adaptors and gel extraction and purification to select for a 400 bp fragment. Kapa Biosystems quantitative PCR was used to assess validated library quantity. The Illumina c-Bot generated clusters on 8 lanes of a flow cell and the Illumina Hi Seq generated 2x100 paired end reads. **Results:** The entire genome of the melanoma was sequenced. Over 90% of the reads had Q scores greater than 30, corresponding to 99.9% base call accuracy. Average coverage of the genome was 30 reads per base. A *BRAF* c.1799A>T (V600E) mutation was detected in 17/29 reads (59%). *BRAF* mutation detection of the original DNA extraction before library preparation showed the same mutation at 57%. **Conclusions:** The protocol described here is a method for next generation sequencing of an entire tumor genome from FFPE. The Q scores and coverage were comparable to sequence from genomic DNA from whole blood or snap frozen tissue. Targeted sequencing, such as of the exome or kinome, when paired with this protocol, would likely provide adequate coverage for somatic mutation detection in FFPE samples.

ST62. High Resolution Melt Analysis (HRMA), a Sensitive Screening Method to Detect Exonic Mutations in Phosphatase and Tensin Homologue (*PTEN*) Gene in Cancers

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Introduction: *PTEN* encodes a tumor-supressor phosphatase capable of dephosphorylating both phospholipids and phosphoproteins. It dephosphorylates phosphotidyl inositol 3,4,5-triphosphate (*PIP3*), thus antagonizing the *PI3K/AKT-PKB* signaling cascade. *PTEN* functions are lost, in at least a subset of most cancers types, due to gene deletion or loss-of-function mutations. These mutations are scattered across all nine exons making mutational analysis for clinical purposes very labor intensive with routinely employed techniques like Sanger sequencing. Here we have explored the use of HRMA analysis as a screening tool to identify the exons with mutations for Sanger sequencing confirmation. **Methods:** DNA from 16 formalin-fixed, paraffin-embedded (FFPE) endometrial tumors was isolated following manual microdissection. PCR to amplify the 9 exons of *PTEN* was performed using 50ng of gDNA template and the amplicon sizes ranged from 147-244 bp. HRMA analysis was performed in Roche LightCycler 480 (Indianapolis, IN). Either DNA from patient samples or cell lines with known *PTEN* mutations was used for optimizing HRMA conditions. HRMA-PCR products from all cases were sequenced bi-directionally using a 3730 Genetic Analyzer (Life Technologies, Carlsbad, CA). Serial dilution studies were performed using positive FFPE CAMA1 cell line (D92H) into negative FFPE HL60 cell line. **Results:** A total of 19 mutations were detected in 16 cases by Sanger sequencing, which included point mutations, insertions and deletions. Exon 5 (encoding phosphatase domain) (6/19 or 32%) and exon 8 (encoding protein-binding domain) (6/19 or 32%) had the highest number of mutations followed by exon 7 (4/19 or 21%), exon 1 (2/19 or 11%) and exon 6 (1/19 or 5%). No mutations were identified in exon 2-4 or 9. HRM was able to detect all the mutations identified by Sanger sequencing exhibiting high sensitivity. However, 9 out of 96 (9.3%) Sanger mutation negative fragments were called variants (mutation positive) by HRM. The sensitivity of HRMA was 10% in serial dilution studies **Conclusions:** High concordance (100%) of mutations detected by HRM and Sanger sequencing demonstrated the reliability and sensitivity of the HRM analysis as a preliminary screening method. Though, there were few false positive calls by HRMA compared to Sanger sequencing, probably due to greater sensitivity of HRMA and/or software settings, there were no false negatives. In a clinical setting, it could be a rapid and cost effective method to identify potentially mutated regions, which could subsequently be confirmed by Sanger sequencing thus avoiding the need to sequence the entire exonic region of *PTEN*.

ST63. Somatic Deletions of the PolyA Tract in the 3'-UTR of *EGFR* Are Common in Microsatellite Instability-High Endometrial and Colorectal Carcinomas

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Introduction: Epidermal growth factor receptor (*EGFR*) is overexpressed in up to 80% colorectal and endometrial carcinomas. The *EGFR* gene contains a polyA tract polymorphism (A13/A14) in its 3'-untranslated region (3'-UTR). Deletions of this polyA tract have been reported in microsatellite instability-high (MSI-H) colonic carcinomas but its impact on *EGFR* expression and downstream oncogenic pathways, such as *KRAS* and *BRAF*, is unclear. Furthermore, 3'-UTR polyA status and its correlation with *EGFR* expression have not been reported in other MSI-H tumors, such as endometrial carcinoma. In this study, we examined the length of the 3'-UTR polyA tract of *EGFR* in both endometrial and colorectal carcinomas and the mutational status of downstream targets of the EGFR signaling pathway. **Methods:** Ninety-eight colorectal carcinomas (36 MSI-H, 22 MSI-low, 40 MSI-stable) and 17 endometrial carcinomas (7 MSI-H and 10 MSI-stable) formalin-fixed, paraffin-embedded patient samples and 30 snap-frozen tissues (15 MSI-H and 15 MSI-stable) endometrial carcinomas were included in this

study. Genomic DNA was isolated and MSI status was determined using a panel of 7 markers (*BAT25*, *BAT26*, *D2S123*, *D5S346*, *D17S250*, *BAT40*, and/or *TGFBRII*). *EGFR* 3'-UTR polyA status was detected by capillary electrophoresis and Sanger sequencing. *EGFR* gene expression and copy numbers were determined by quantitative qPCR reaction. *KRAS* and *BRAF* mutation status was analyzed by pyrosequencing. **Results:** The 3'-UTR polyA tract was deleted in 18 of 23 (78%) MSI-H versus 0 of 24 MSI-stable (MSS) endometrial carcinomas (P= .001). Similar observations were seen in colorectal carcinomas in which 29 of 36 (81%) MSI-H, 1 of 62 (1.6%) MSI-low (MSI-L) and none of the MSI-stable (MSS) tumors harbored 3'-UTR polyA deletions (P=.001). A moderate increase in *EGFR* mRNA level was observed in endometrial carcinomas with 3'-UTR polyA deletions versus those with wild-type polyA tract. Amplification of the *EGFR* gene was not observed by qPCR based copy number analysis. Deletions in polyA tract do not seem to affect the frequency of *KRAS* and *BRAF* mutations. **Conclusions:** Deletions of *EGFR* 3'-UTR polyA is frequent in endometrial and colorectal carcinomas. Deletions in the 3'-UTR polyA tract of *EGFR* were confined almost exclusively to MSI-H tumors. PolyA deletion does not seem to affect the frequency of *KRAS* and *BRAF* (V600E) mutations.

ST64. Sensitive and Rapid Detection of *KRAS* Mutations by Peptide Nucleic Acid (PNA) Clamp PCR in Endometrial Cancer

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Introduction: Somatic *KRAS* mutations are found in a wide variety of malignancies. Assessment of *KRAS* mutation status at codons 12 and 13 provides valuable guidance for cancer management, particularly with respect to targeted therapy. Direct sequencing from macro- or microdissected tumor cells has been traditionally used to identify mutations but this method has a low sensitivity. Peptide Nucleic Acid (PNA) clamp PCR is one approach to increasing sensitivity by enriching for mutant alleles. This study compares the sensitivity and specificity of PNA clamp PCR compared to direct sequencing for the detection of *KRAS* mutations in mucinous carcinoma (MC) and endometrioid carcinoma (EC), endometrioid carcinoma with significant Mucinous differentiation (ECMD) and serous carcinoma (SC) of the uterus. **Methods:** With IRB approval, 52 cases were selected from archives database at Women and Infants Hospital (Providence, RI). There were two cases with positive lymph nodes. Genomic DNA was extracted from microdissected formalin-fixed, paraffin-embedded sections and subjected to both direct sequencing and PNA clamp real-time PCR using a ΔCt value calculation to determine the mutation status. Sequencing of the PNA PCR product was used to identify the specific *KRAS* mutation as well as resolve discrepancies with direct sequencing results. **Results:** *KRAS* mutations were detected in 23 cases (23/52, 44%) including 7 of 9 (78%) MC, 11 of 16 ECMD (69%), 4 of 16 EC (25%), and 1 of 11 SC (9%). Statistically significant differences in *KRAS* mutational status were noted between MC vs EC ($P<0.05$), ECMD vs EC ($P<0.05$), MC vs. SC ($P<0.01$), and ECMD vs. SC ($P<0.01$, Fisher's exact test). Initially, 13/52 cases tested positive by PNA clamp PCR and negative by direct sequencing. Four of these cases may represent positive cases not detected by direct sequencing, four cases appear to be false positives and five cases are still under investigation. No cases were direct sequencing positive and PNA clamp PCR negative. PNA clamp PCR compared to direct sequencing is 100% sensitive and 75% specific. **Conclusions:** Compared to direct sequencing, PNA clamp PCR is able to rapidly screen for the presence or absence of *KRAS* mutations with high sensitivity. The specific point mutation in codon 12 or 13 can then be identified in positive patients by sequencing the PNA PCR product. This reduces the amount of labor intensive sequencing needed to identify these mutations.

ST65. Novel Mutations in *KRAS* Codons 12 and 13 Are Likely Informative Regarding Cetuximab or Panitumumab Treatment Choices

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Introduction: Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer death in the United States. Approximately 101,700 people in the United States will likely be diagnosed with colorectal cancer in 2011. Survival of metastatic colorectal cancer patients has nearly doubled in the past decade. This improvement is mainly due to the development of new combinations of standard chemotherapy and also to the introduction of monoclonal antibodies such as cetuximab or panitumumab against epidermal growth factor receptor (*EGFR*) or against vascular endothelial growth factor. Several studies demonstrate that patients with metastatic colorectal cancer, who have a *KRAS* mutation in codon 12 or 13, do not derive benefit from treatment with cetuximab or panitumumab. Additionally, the use of cetuximab or panitumumab therapies may result in increased toxicity. A *KRAS* mutation is present in approximately 40% of metastatic colon cancer tumors. **Methods:** Our laboratory, participating in the CERGEN study (Comparative Effectiveness Research in Genomics and Personalized Medicine for Colon Cancer funded by the National Cancer Institute) tested for variants in codons 12 and 13 in the *KRAS* gene in 367 individuals diagnosed with metastatic colorectal cancer. DNA from these individuals was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue on unstained slides; to aid in the enrichment of tumor-originating DNA, the specific location of tumor tissue on the slide was confirmed microscopically by a pathologist on a companion H&E slide, circled and percent tumor indicated. We used Sanger sequencing to determine genotypes using primers flanking the start and end of exon 2 (in which codons 12 and 13 are embedded). **Results:** Here we report two novel *KRAS* mutations (as determined by inspection of the COSMIC data base on June 1, 2011): c.36_47dupTGGCGTAGGCAA and c.38_39insTGG; additionally a previously unreported complex mutation was detected, c.[38G>A(+)40G>A]. **Conclusions:** All of these mutations are predicted to disrupt the phosphate-binding P-loop in the *KRAS* protein (encoded by codons 10-17), likely resulting in autoactivation of the *KRAS* protein. Due to this autoactivation, cetuximab or panitumumab are likely to be ineffective treatments.

ST66. *KRAS* Mutation Analysis Is a Good Adjunct to Cytologic Evaluation of Pancreatic Cyst Fluids

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Introduction: Cystic lesions of the pancreas have increasingly been detected due to advances in diagnostic endoscopy. Diagnostic approach includes cytologic examination and tumor marker analysis in cyst fluid. However this approach has its limitations and may further benefit from molecular testing. The objective of this study is to evaluate the utility of *KRAS* mutation analysis in pancreatic cyst samples. **Methods:** From 2008 to 2011, we prospectively evaluated 44 patients (22 male, 22 female, median age 56.5) with pancreatic cyst(s) who underwent endoscopic ultrasound (EUS) guided fine needle aspiration (FNA) of cyst fluid. The median follow-up period was 92 days (range: 14 to 540 days). A total of 47 FNA samples were collected. Based on the cytology diagnosis, the cases were categorized into four groups: non-diagnostic (group 0, 28%), negative (group 1, 53%), atypical/suspicious (group 2, 15%), and positive for malignancy (group 3, 4%). *KRAS* mutation on codon 12 and 13 was tested by quantitative PCR (sensitivity 0.1%). DNA quality/quantity was evaluated using multiple methods. Cyst fluid CEA and amylase levels were also measured. Clinical follow-up data was obtained. **Results:** Forty-six samples (98%) had adequate quality for PCR. *KRAS* mutation was detected in 39% (18/46) of cases – 46% (6/13), 21% (5/24), 71% (5/7) and 100% (2/2) in group 0, 1, 2 and 3, respectively. Surgical resection was performed in 5 patients – a mucinous cystic neoplasm with focal low grade dysplasia (group 0), a mucinous cystic neoplasm and a pseudocyst (group 1) and 2 intraductal papillary mucinous neoplasms (group 2). In group 3, patients were treated with neoadjuvant chemotherapy. The remaining patients had either observation or no follow-up data. Difference in cyst fluid CEA level was observed (249.1 vs. 25.1 ng/ml, median value), but no difference in quality or quantity of DNA and cyst fluid amylase levels were found between *KRAS* mutant and wild-type cases, respectively. **Conclusions:** Among patients with negative cytology and wild-type *KRAS*, 18 of 19 showed no evidence of neoplasm. By contrast, at least 4 of 7 patients with atypical/positive cytology and mutant *KRAS* had a tumor. *KRAS* mutation was identified in 30% (11/37) of cytologically non-diagnostic or negative cases. Thus *KRAS* mutation testing can serve as a good adjunct to cytology. Further studies with additional molecular markers and long-term clinical follow-up are needed to assess the clinical specificity of mutation testing.

ST67. Oncogene Mutation Analysis of Cholangiocarcinoma Using the OncoCarta Panel and MassArray Technology

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Introduction: Cholangiocarcinomas (CCA) are rare and often lethal cancers of the biliary tract. Despite technologic advancements over the past decade, little is known about the somatic changes that occur in these tumors. The goal of this study was to use MassArray technology to identify common mutations in CCA specimens that could aid in the development of assays for early detection and/or provide potential therapeutic targets for patients diagnosed with CCA. **Methods:** Eight unstained, 10 micron sections were cut from formalin-fixed, paraffin-embedded tissue blocks from 100 CCAs. DNA was extracted from areas comprising >20% tumor as determined by a board certified pathologist. Specimens were then evaluated using the Sequenom OncoCarta™ Mutation Profiler Panel (Sequenom Inc., San Diego, CA). This MALDI-TOF mass spectrometry SNP genotyping panel evaluates 19 oncogenes for 238 somatic mutations. **Results:** Twenty-seven of the 100 CCA specimens tested in this study

harbored a mutation in 1 (n=24) or 2 (n=3) oncogenes. The 30 mutations included alterations of the following oncogenes: *KRAS* (n=12), *PIK3CA* (n=4), *MET* (n=4), *EGFR* (n=3), *BRAF* (n=2), *PDGFRA* (n=2), *ERBB2* (n=1), *NRAS* (n=1) and *RET* (n=1). Mutations were identified in 11 of 31 (35%) extrahepatic CCAs and 16 of 69 (23%) intrahepatic CCA's. MassARRAY also detected what appeared to be a homozygous *EGFR* G719S mutation in one specimen. However, fluorescence *in situ* hybridization testing demonstrated that this specimen had *EGFR* amplification of the G719S mutated allele. **Conclusions:** MassARRAY technology can be used to detect mutations in a wide variety of oncogenes using paraffin-embedded tissue. Twenty-seven percent of the evaluated CCAs harbored a mutation within at least one of the panel oncogenes. Because most of these mutations occur in oncogenes that encode proteins for tyrosine kinase receptors or proteins involved in currently targeted signaling pathways, clinical testing of CCA for these mutations may drive personalized therapy selection for these tumors in the future. The variety of mutations detected suggests that a multiplexed mutation detection approach is necessary for both diagnostic and theranostic algorithms for CCA.

ST68. Prevalence of Microsatellite Instability and *MLH1* Promoter Methylation Status among Hispanic and Caucasian Patients with Colorectal Cancer in New Mexico

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Introduction: Microsatellite instability (MSI) occurs due to defective DNA mismatch repair and is associated with Lynch Syndrome (LS) as well as a proportion of sporadic colorectal cancer. LS-associated colorectal cancer (CRC), comprises approximately 5% of all CRC. The MSI in greater than 2 markers (MSI-H) is found in about 95% of LS associated CRC and in approximately 20% of sporadic CRC. Likewise, *MLH1* promoter hypermethylation is found in sporadic CRC and not in LS-associated cancers. Therefore, MSI and *MLH1* promoter methylation status testing are useful as screening tools to identify individuals with potential LS allowing them to benefit from increased cancer surveillance. There is only limited data in English literature addressing the prevalence of MSI-H in Hispanic patients with CRC versus Caucasian patients with CRC. In this study, we analyzed the MSI and *MLH1* promoter methylation status in Hispanic and Caucasian patients with CRC. **Methods:** MSI analysis was performed on DNA extracted from paraffin-embedded tumor tissue in parallel with corresponding benign tissue on 21 patients including 12 Caucasians and 9 Hispanics. The age of the patients included in this study ranged from 25 to 85, with a median age of 49. The analysis was performed using Promega MSI kit (Marker panel: BAT-26, BAT-25, NR-21, NR-24, MONO-27) followed by capillary electrophoresis for sizing on the ABI 3130 Genetic Analyzer. *MLH1* promoter methylation analysis was performed by pyrosequencing analysis of bisulfite-treated DNA on the PyroMark Q24 System by Qiagen. **Results:** Seventeen of 21 cases including 7 Hispanic (78%) and 10 Caucasians (83%) showed MSI-H, defined by 2 or more loci demonstrating microsatellite instability. The remaining 4 patients were microsatellite stable, defined by absence of instability in any of the 5 loci analyzed. *MLH1* promoter hypermethylation was identified in 7 cases, including 2 (29%) Hispanics and 5 (71%) Caucasians with MSI-H. None of the MSI stable cases demonstrated *MLH1* hypermethylation. **Conclusions:** Our results indicate that the prevalence of MSI-H is similar among New Mexican Hispanic and Caucasians patients. This finding further confirms the results of prior studies. Although the number of cases in our study is low, the relatively high incidence of *MLH1* promoter hypermethylation among Caucasian group with MSI-H compared to Hispanic group, may suggest that LS-associated MSI may be more common in individuals of Hispanic origin.

ST69. Discrimination of Normal Lung, Squamous Cell Carcinoma, and Adenocarcinoma Using a Novel Panel of Five MicroRNAs to Analyze Archival Tissues

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Introduction: Noon-small-cell lung carcinoma (NSCLC) can be differentiated into squamous cell carcinoma (SCC) and adenocarcinoma (Ad), which have different treatments and prognosis. Therefore it is critical to establish an accurate pathologic diagnosis. Among the current available diagnostic tools, while this process can be aided by expert morphologic and immunohistochemical analysis, molecular characterization offers higher level of resolution. There is increasing evidence that different cell types have distinct microRNA (miRNA) expression profiles. Since miRNA can be efficiently extracted from formalin-fixed, paraffin-embedded (FFPE) archival tissues, we tested the ability to distinguish between normal lung (NL) and the malignant counter parts, SCC and Ad. **Methods:** With approval by the IRB for analysis of de-identified specimens, 61 independent FFPE lung specimens were analyzed for miRNA expression. Microarray analysis (Agilent) was performed on 16 specimens of different subtypes to determine the whole genome miRNA profile. Total RNA was extracted from paraffin blocks using Recover All total nucleic acid extraction kit (RNA protocol) from Ambion. RNA were labeled and hybridized to the microarray according to the manufacturer's procedure. The median of the log2 transformed raw signal intensity data was normalized and processed through an invariant normalization. Among 34 miRNA differentially expressed in NL, SCC, and Ad, the expression levels of the 9 with the highest level of discrimination were verified by Q-PCR (Applied BioSystems) with additional specimens. A final panel of 5 miRNA (miR-205, -26b, -486-5p, -451, -21) was further selected to complete the Q-PCR analysis for all samples. **Results:** Microarray data demonstrated proper clustering of NL, Ad, and SCC subgroups with distinctive expression patterns. miR-451 and miR-486-5p were down regulated in the malignant specimens regardless the subtype. miR-21 was increased in cancer specimens as previously identified. These three microRNAs can be used to distinguish NL from SCC and Ad. Expression of miR-205 was dramatically increased in SCC specimens as previously reported. miR-26b expression was modestly reduced only in SCC in comparison to Ad and NL. Combining the miR-205 and miR-26b expression data enabled us to differentiate SCC from Ad. These results were highly reproducible between different experiments using the delta Cp method to normalize total input RNA and assay conditions. **Conclusions:** We identified a panel of 5 miRNAs, including novel species, that can be used to distinguish NL from tumor and SCC from Ad. These findings demonstrate the potential clinical utility to distinguish between poorly differentiated cases of SCC and Ad.

ST70. Comparison of Somatic Point Mutation Detection Methods in Colorectal Carcinoma: Addressing the Challenges of Analytical Sensitivity

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Introduction: The identification of somatic mutations affecting signal transduction of the epidermal growth factor receptor (*EGFR*) pathway, including activating missense mutations of *KRAS* and *BRAF*, is important for clinical decisions regarding targeted therapy in colorectal cancer, since these mutations confer resistance to anti-*EGFR* monoclonal antibodies. Reliable detection of somatic mutations with an analytic sensitivity <10% is challenging, and defining a minimum level at which a patient may still derive a positive benefit from targeted therapy is unknown. The aims of this study were to determine the limits of detection for two primer extension-based assays using clonal, mutation-specific reagents and compare performance between the two assays on formalin-fixed, paraffin-embedded (FFPE) colorectal adenocarcinomas. **Methods:** One primer extension assay is based upon the method of Lurkin et al (PLoS ONE. 2010, 5:e8802) in which multiplex PCR amplification of the relevant *KRAS* and *BRAF* exons is followed by single nucleotide probe extension using the SNaPshot™ Multiplex Kit (Applied Biosystems, Foster City, CA) and detection of the products by capillary electrophoresis. The second assay, the Mutector kit (Applied Biosystems), follows the amplification step with a primer extension step using proprietary shifted termination assay reagents. *KRAS* and *BRAF* mutation-specific DNA fragment control reagents were PCR generated and cloned using the QuickChange® Lightning Site-Directed Mutagenesis kit (Stratagene) for plasmid and cell-based control reagents. All laboratory-derived positive control reagents were sequence verified. The two assays were used to evaluate a panel of colorectal adenocarcinomas FFPE tissue specimens for which the *KRAS* and *BRAF* mutation status was previously determined. **Results:** The assays showed 100% concordance between each other and 94% against the prior determination (also a primer extension based method) for the detection of the most common *KRAS* mutations from 17 patient specimens. This failure of both assays to detect a low-abundance G12D mutation in one tumor with infiltrating histology possibly emphasizes the need for more sophisticated tumor cell collection such as laser capture microdissection. The relative proportions of the mutant peak height to the wild-type peak height give an estimated analytical sensitivity of ~10%. The analytic sensitivity of both assays using dilution of *KRAS* mutation-specific clones was higher. **Conclusions:** The low analytical sensitivity of primer extension-based detection of somatic *KRAS* mutations denoted in this analysis demonstrate the need to further optimize these methods in order to address the important clinical issue of defining a lower threshold for reporting mutation-positive specimens.

ST71. Molecular Mechanisms that Contribute to Heterogeneous Scanty Staining for *MSH6* in Colorectal Carcinoma

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Introduction: IHC for MMR proteins is a well-accepted approach to evaluate patients for Lynch Syndrome (LS). Rare *MLH1*- and/or *PMS2*-deficient CRCs as well as post-treatment MMR proficient rectal cancers can show only scanty staining (i.e., near complete loss of staining) for *MSH6*. We have observed a scanty staining pattern with *MSH6* antibody in a small proportion of our cases (9/420) that poses problems for

interpretation. This study aimed at elucidating the molecular mechanisms that contribute to this aberrant pattern of staining. **Methods:** DNA was extracted from 10 formalin-fixed-paraffin-embedded (FFPE) tissue sections from colorectal tumors using the Qiagen DNeasy Tissue kit (Qiagen, 69504) from these 9 cases. The coding exon containing a mononucleotide repeat sequence, an 8-cytidine repeat, (C)8, was amplified and sequenced. DNA fragments with frameshift in the C8 region were cloned into pCR4 TOPO vectors (Invitrogen, Carlsbad, CA). DNA from colonies was amplified and subjected to direct DNA sequencing analysis. **Results:** Of the 9 cases, 4 cases had additional, unstable alleles in the C8 region in *MSH6*. We cloned PCR fragments into TOPO vectors for two cases. We observed C8 in about half of the colonies (45% (5/11) and 43% (3/7)). The remaining colonies had C7, C9 or C10 repeats, leading to frameshifts. In these 4 cases, *MLH1* and/or *PMS2* were absent in the tumor by IHC (a germline *PMS2* R315X mutation was identified in one case). We did not observe any frameshift in the other 3 cases (MMR proteins were present). Two cases could not be studied because of insufficient amount of material. **Conclusions:** Secondary mutations in the mononucleotide repeat sequence, C8, in the *MSH6* coding region explain the scanty staining for *MSH6* in some *MLH1*- and/or *PMS2*- deficient cancers.

ST72. *IDH1* and *IDH2* Mutation Detection by Melting Curve Analysis in Brain Specimens

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Introduction: Mutations in the genes encoding isocitrate dehydrogenase 1 (*IDH1*) and 2 (*IDH2*) have been found in a majority of grade 2 and 3 astrocytomas as well as oligodendrogliomas and some glioblastomas, while the mutations are absent or very uncommon in tumors outside the central nervous system. Techniques used to assess IDH mutations have included pyrosequencing, Sanger sequencing, immunohistochemistry, and PCR-RFLP. Melting curve analysis after real-time PCR has also previously been applied to IDH testing in brain tissues and shown to correlate with results from Sanger sequencing. This study compares newly designed *IDH1* and 2 primers and probes to previously reported ones for use in melting curve analysis as a tool for detection of IDH mutations in glial neoplasms. **Methods:** Forty-two formalin-fixed, paraffin-embedded tissues were analyzed, including neoplastic brain lesions (n=20), benign brain tissue (n=10), and non-nervous system tissue (n=12). Nucleic acids were extracted using Qiagen QIAamp DNA FFPE Tissue kits. DNA was amplified and subjected to melting curve analysis using previously published *IDH1/IDH2* primers and FRET probes on a Roche LightCycler, following the authors' protocol. The same sample DNA was then analyzed using an optimized protocol and unique primers and LightCycler probes designed in our lab. In both assays mutations (heterozygous) manifested as second peaks in the melt curve. Parameters addressed when comparing the assays included robustness of DNA amplification and ability to discriminate wild type from mutant based on differences of melting temperatures (TM). All mutations detected by melting curve analysis were verified by Sanger sequencing of forward and reverse strands. **Results:** More robust amplification was achieved with the newly designed primers compared to those used in the published assay. Overall there was no significant difference in the ability of the previously published and newly designed FRET probes to differentiate between mutant and wild type based on the TM spread. DNA sequencing was in agreement with mutation assessment by melting curve analysis. **Conclusions:** Our results show further optimization of melting curve analysis as a sensitive and viable method for the analysis of *IDH1* and *IDH2* mutations in glial neoplasms. *IDH1* and *IDH2* melting curve analysis represents a powerful adjunct method for diagnosis of glial neoplasms from brain biopsies in complex or otherwise challenging cases.

ST73. Comparison of Peptide Nucleic Acid (PNA)-Mediated PCR Clamping, COLD-PCR, and Direct Sequencing in Detection of *PIK3A*, *BRAF* and *KRAS* Mutations in Colorectal Cancers

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Introduction: *KRAS*, *BRAF*, and *PIK3CA* mutation testing before anti-epidermal growth factor receptor therapy of metastatic colorectal cancer (CRC) has become important. However, considerable uncertainty exists as to which methods for detection can be applied in a reproducible, sensitive, and simple manner in the routine diagnostic setting. **Methods:** We compared the detected rate of *KRAS*, *BRAF*, and *PIK3CA* mutations in 92 routine paraffin tissues of CRC specimens by three discrete methods of direct sequencing, co-amplification-at-lower denaturation-temperature PCR (COLD-PCR) and peptide nucleic acid (PNA)-mediated PCR. **Results:** The *KRAS*, *BRAF*, *PIK3CA* mutations were detected 21%, 3%, and 1% by direct sequencing. The use of COLD-PCR raised the percentage of mutated CRCs by 6%, 4%, and 2% for *KRAS*, *BRAF*, and *PIK3CA* compared than the detection rate of regular PCR followed by direct sequencing (p = 0.031, p = 0.250, and p = 0.250, respectively). PNA-mediated PCR clamping also increased the percentage of *KRAS*, *BRAF*, and *PIK3CA* mutation up to 7%, 2%, and 6% compared than the detection rate of regular PCR followed by direct sequencing (p = 0.039, p = 0.250, and p = 0.031, respectively). **Conclusions:** COLD-PCR and PNA-mediated PCR clamping method are similarly more sensitive for the clinical diagnosis of *KRAS*, *BRAF*, and *PIK3CA* mutations.

ST74. Evaluation of Real-Time PCR Assay Options for Detection of *MGMT* Promoter Methylation

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Introduction: O6-methylguanine-DNA-methyltransferase (*MGMT*) gene silencing by promoter hypermethylation is associated with improved survival and increased response in patients with glioblastoma to alkylating agents, such as temozolomide. Methylation-specific PCR (MSP) was used in several clinical trials that established *MGMT* methylation as a positive predictive and positive prognostic marker. In addition to a lengthy bisulfite conversion of genomic DNA, MSP methodology requires two rounds of nested PCR followed by gel electrophoresis. This is a labor-intensive assay that can result in amplicon contamination and be difficult to score since it is entirely non-quantitative. **Methods:** Two sets of previously published primer and probe sequences for *MGMT* and ACTB (TaqMan and MGB-TaqMan) were used along with two different commercially-available PCR master mixes to develop a robust real-time, quantitative methylation-specific PCR (qMSP) assay that would allow for detection of methylated *MGMT* promoter from genomic DNA isolated from formalin-fixed, paraffin-embedded (FFPE) tumor tissue. All DNA samples were isolated from FFPE tissue and subjected to the EpiTect Bisulfite kit (QIAGEN). The bisulfite-treated DNA was then subjected to real-time PCR amplification on a 7500 Fast Real-time PCR System (Life Technologies) using either TaqMan Universal PCR Master Mix (Life Technologies) or EpiTect MethyLight master mix (QIAGEN). Human genomic DNA controls (methylated and unmethylated) were used to assess assay performance and to produce standard curves. **Results:** With both primer/probe sets the EpiTect MethyLight master mix outperformed the TaqMan Universal PCR master mix with respect to sensitivity. This can be observed by the detection of diluted control samples and FFPE samples that were either not detected with the Universal master mix or detected at much lower levels than the EpiTect master mix. When both sets of primer/probes were compared using the Epitect master mix, PCR efficiencies were all near 100%, although the linear range of the assay with the *MGMT* Taqman probe did not extend as low as the assay with the *MGMT* MGB-TaqMan probe. **Conclusions:** Methylation-specific real-time PCR assays have an increased potential to serve as clinical diagnostic assays due to their quantitative nature and the decrease potential for PCR contamination. The qMSP methods described in the literature are much less robust in our hands when testing DNA from FFPE tissues instead of fresh tissue. Modifying qMSP protocols by using an alternative PCR master mix results in a marked increase in assay performance

ST75. Validation of the Agilent High-Resolution Array-CGH Platform and Comparison of aCGH to FISH in Diagnostic Evaluation of Cutaneous Melanoma

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Introduction: Benign melanocytic nevi and cutaneous malignant melanomas can be difficult to distinguish from each other by routine microscopic analysis. Recent evidence has suggested that cytogenetic analysis may be a useful ancillary method for diagnostically difficult melanocytic proliferations. Melanomas tend to carry recurrent unbalanced chromosomal aberrations, while melanocytic nevi usually do not contain detectable chromosome aberrations by CGH analysis, except for a subset of Spitz nevi. Therefore, we validated Agilent Human 244K aCGH platform for clinical diagnosis of melanocytic tumors, and compared aCGH results with a four-probe FISH assay in diagnosis of melanoma. **Methods:** We studied 33 FFPE samples from our melanocytic tumor archive, including 28 melanomas and 5 benign nevi. The aCGH raw data were analyzed by Agilent Cytogenomics software and a list of aberration findings (CNVs, Copy Number Variations) was generated for each sample. CNVs identified in regions contained no annotated genes or fully overlapped by known benign CNVs, were filtered out from the aberration finding list. The remaining CNVs with more than 100 probes involved (i.e. >700Kb in size) were considered as solid findings and thus weighed in favor of the diagnosis of melanoma. Four-probe FISH assay (*RREB1*/*MYB*/*CEP6* and *CCND1*), approved by NYS as an adjunct clinical diagnostic method for Cutaneous Melanoma, was performed on 22 melanoma samples with sufficient materials. aCGH findings and FISH results were interpreted independently in a blinded fashion. **Results:** No solid finding was noticed in any of the 5 benign nevi by aCGH analysis. In contrast, significant unbalanced genomic aberrations were revealed in 27 melanomas

(96%), 26 of which exhibited complex unbalanced genomic aberrations. Positive results were obtained in 15 melanomas (70%) by four-probe FISH analysis, and negative results in five cases; two cases were uninformative. In addition, major aCGH findings of focal homozygous deletions/amplifications were also verified by FISH. The overall concordance in aberration detection between the two methods was 95%. **Conclusions:** Copy number analysis of FFPE tumor samples by aCGH is a sensitive and reliable method in diagnosis of melanoma. The evaluation of the whole genome by aCGH is in particular valuable for cases in which the histopathologic features alone do not permit definitive diagnosis and the four-probe clinical FISH assay is uninformative (normal results, equivocal findings due to poor quality of the FFPE section, or failure).

ST76. Targeted Screening of Colorectal Cancers for a Novel *BUB1B* Mutation

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Introduction: The *BUB1B* gene encodes the spindle-assembly checkpoint regulator protein, BUBR1. Mutations in the *BUB1B* gene have been reported in cases of mosaic variegated aneuploidy syndrome, an extremely rare cancer susceptibility syndrome with autosomal recessive inheritance. Symptoms of mosaic variegated aneuploidy include growth deficiency, microcephaly, and cancer (often by age 2). In a recent case report, a homozygous, intronic *BUB1B* mutation resulting only in reduced *BUBR1* expression was discovered in a patient with a history of multiple gastrointestinal cancers, but lacking the typical clinical features of mosaic variegated aneuploidy. This patient was initially suspected of having Lynch syndrome, but cytogenetic testing indicated mosaic variegated aneuploidy syndrome. A population of 590 unaffected individuals was screened for this mutation, resulting in a single heterozygote. In order to further elucidate a potential significance for this *BUB1B* mutation beyond the proband described, we tested DNA samples from two groups of colorectal patients to see if the frequency of this mutation might be higher in a more targeted population **Methods:** DNA samples were isolated from archived FFPE tissue of two groups of colorectal cancer patients. A "*KRAS* negative" group consisted of 23 cases that had been submitted for *KRAS* mutation testing and found to be negative for the codon 12 and 13 mutations and a "MMR intact" group consisted of 19 colorectal cancer patients suspected of Lynch syndrome but found not to be deficient in *MLH1*, *MSH2*, *MSH6*, or *PMS2* by immunohistochemistry. A TaqMan genotyping assay was designed to detect the c.2386-11A>G mutation and corresponding wild-type sequences in *BUB1B*. Wild-type and mutant oligonucleotide controls were constructed to confirm assay performance. **Results:** The TaqMan assay was able to discriminate between homozygous wild-type, heterozygous and homozygous c.2386-11A>G control samples. All 23 DNA samples from the "*KRAS* negative" group and all 19 samples from the "MMR intact" group were found to be wild type with respect to the c.2386-11A>G mutation. **Conclusions:** The absence of the *BUB1B* c.2386-11A>G mutation in the two groups of colorectal cancer patients examined in this study indicate that this mutation is not commonly correlated with colorectal cancer. While larger studies may show additional cases of gastric or colorectal cancer resulting from mosaic variegated aneuploidy syndrome and *BUB1B* mutations, these cases would be expected to be infrequent.

ST77. Highly Sensitive Molecular Assay for the Detection of Circulating Cell-Free *BRAF* V600E Tumor DNA in the Serum and Plasma of Melanoma Patients

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Introduction: V600 mutations of the *BRAF* gene have been described in approximately 8% of all solid tumors, with the frequency exceeding 50% in melanomas. Several *BRAF*-specific inhibitors are currently undergoing Phase II/III trials in late stage V600E *BRAF*-positive melanoma patients with promising results, such as vemurafenib, which has shown better than 80% response rate. However, non-invasive and highly sensitive techniques for *BRAF* mutation detection and monitoring therapeutic efficacy are currently lacking. This study describes the development and validation of a sensitive and highly specific molecular assay for the detection of *BRAF* V600E mutations in circulating cell-free (cf) tumor DNA in plasma/serum utilizing the MassARRAY® MALDI-TOF platform. **Methods:** Plasma and serum DNA were isolated from Stage IV melanoma patients and healthy volunteers. Plasma spiked with DNA extracted from cell lines containing either the wild-type (wt) *BRAF* gene (PANC1) or the *BRAF* V600E mutation (SK-MEL28, HT29), served as controls. Cell line DNA was fragmented to 200-1000bp in size prior to spiking to emulate circulating cfDNA. PCR amplification with concurrent digestion of wt*BRAF* using thermostable TspRI endonuclease resulted in preferential amplification of mutant *BRAF* DNA. The amplified DNA was then subjected to single base mutation detection using iPLEX® technology on the MassARRAY® platform (Sequenom Inc. San Diego, CA). **Results:** The assay was shown to reliably detect V600E *BRAF* in plasma spiked with 50pg/mL of mutant cell-line gDNA (equivalent to 3-5 copies of the V600E *BRAF* gene per reaction). The presence of up to 500ng of additional wild-type genomic DNA per reaction was well tolerated and did not affect the detection of the mutant allele. Clinical performance was assessed on a blinded panel of plasma samples and tissue biopsies from 31 stage IV melanoma patients. Fourteen of 31 patient tumors had V600E mutant DNA present. *BRAF* V600E mutations were detected in circulating cfDNA extracted from the plasma of 11/14 patients, demonstrating 79% sensitivity (95%CI 0.52-0.94, NPV 0.64-0.95). Plasma matched to all of the 17 wt*BRAF* tumors tested negative, demonstrating 100% specificity (95%CI 0.82-1.00, PPV 0.74-1.00). Serum from 50 healthy volunteers all tested negative for V600E cf*BRAF*. **Conclusions:** We present a novel method to specifically detect extremely low levels of circulating cell-free mutant *BRAF* DNA of tumor origin in patient plasma or serum. This test can be potentially used to stratify patients to *BRAF* inhibitor therapy and to monitor tumor status non-invasively following treatment in Stage III/IV melanoma patients.

TECHNICAL TOPICS

TT01. Two Complementary and Scalable PCR-based Workflows Enable Next Generation Sequencing of Cancer-Associated Genes in FFPE Tumor DNA

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Introduction: Advances in next generation sequencing (NGS) technologies have enabled an unprecedented view of the genetic events and molecular pathways associated with cancer initiation and progression. These technologies, combined with the armament of nearly 1000 anti-cancer drugs approved or in development, provide a compelling opportunity to empower cancer personalized medicine by matching patient-specific molecular abnormalities and dysfunctional cellular pathways with targeted therapies. NGS of clinical specimens, however, has been largely restricted to analyses of high quality DNA from fresh frozen tissues rather than the modified DNA from FFPE samples that represent the majority of tumor biopsies. In addition, the heterogeneous nature of cancer specimens requires "deep" sequencing (eg, 5,000-10,000X read depth) to reveal low abundance mutations, such as those involved in drug resistance, that may be clinically relevant. **Methods:** Two FFPE-compatible PCR-based enrichment panels were developed. The first was a multiplexed PCR assay that targeted 35 amplicons in 16 cancer genes, including the most common mutations in the *MAPK/ERK* and *PI3K/AKT* pathways. The second included nearly 1000 amplicons from 52 cancer genes. Primers were designed to avoid known SNPs, repetitive sequences, and psuedogenes whenever possible, and included adaptor sequences to enable direct sequencing on either the Ion Torrent PGM or the Illumina GAIIx. **Results:** We optimized target enrichment methods based on PCR that are scalable from dozens to thousands of amplicons, and are compatible with NGS of FFPE DNA. These methods can identify "actionable" mutations in 50 or more cancer-associated genes, including both oncogenes and tumor suppressor genes, and can enable the detection of nearly all of the known mutations from these genes indexed in databases such as COSMIC. The procedures can detect as few as 1% to 2% variants using PCR enrichment strategies that, in the case of the Illumina GAIIx, can be barcoded at up to 36 samples per lane. Identified mutations can be confirmed using one of several orthogonal methods, including an independent target enrichment method that is compatible with the Ion Torrent PGM. **Conclusions:** The combination of two NGS approaches can accommodate both large-scale, whole exon mutation assessments across 48-96 samples per run, as well as "hotspot" mutation analyses across 15-20 genes with a rapid turnaround time (~1 week). Consequently, this strategy addresses current unmet needs for unbiased and high sensitivity mutation detection in FFPE tumor specimens, and can accelerate both basic and clinical cancer research.

TT02. Detection of Expression Levels of LincRNAs in Human Whole Blood Over Time: A High-Potential Future Diagnostic Tool?

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Introduction: With a size of up to several kilobases, long intergenic non-coding RNAs represent the larger counterparts to small regulatory RNAs like microRNAs. Former scientific studies suggest that lincRNAs play an essential role in a diverse set of functions like the regulation of chromatin states or their involvement in epigenetic inheritance. Links to many diseases like cancer are assumed, too. Therefore, we were interested in the detection of these regulatory RNA species in human blood that may become useful in the future for many diagnostic applications. We have successfully provided evidence for their detectability in blood and also compared the transcription levels over a period of time from 2 to 48 hours after blood collection for 12 different lincRNAs. **Methods:** Blood was collected in PAXgene® Blood RNA tubes from consented healthy adults. Afterwards, RNA was isolated manually using the PAXgene

Blood miRNA protocol. The qualitative analysis of the extracted RNA was performed using a Spectramax photometer to measure the optical density and an Agilent Bioanalyzer to check for the RNA integrity. Quantitative analysis of the purified RNA was accomplished by duplex qRT-PCR using 18S rRNA as an internal standard. In addition, long-range PCRs were conducted in order to demonstrate the full length of the transcripts. **Results:** Most lincRNAs were well detectable in the blood samples with only few exceptions like XIST and TSIX. HAR1A, NEAT1 and SNHG8 displayed constant CT-values for all donors at each point in time. In contrast to this, expression levels of other lincRNAs like HOTAIR fluctuated over the assigned time frame. The missing signal of, for example, XIST is easily explicable as this lincRNAs is involved in the X-chromosome-inactivation only during the embryonic stage. In general, false-positive signals from gDNA contamination can be excluded due to a low overall gDNA contamination and tests with a second DNA digest. Furthermore, with the help of long-range PCRs the existence of full-length transcripts was verified. **Conclusions:** To our knowledge, the analysis of lincRNA expression profiles in human whole blood was performed for the first time. This provides researchers with a new powerful tool for the development of new biomarkers. The PAXgene Blood miRNA kit and the described PCR assays are for research use only. Not for use in diagnostic procedures.

TT03. Direct Molecular Characterization and Enumeration of Circulating Tumor Cells in Lung Cancer Blood by Digital Sample Enrichment and RT-CastPCR Technology

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Introduction: Enumeration and molecular characterization of circulating tumor cells (CTCs) promise to be valuable for cancer diagnosis, survival prognosis, and treatment guidance. However, current methods require extensive enrichment process before analyzing rare CTCs in human blood. **Methods:** We reported a new approach for direct CTC detection in blood samples without cell sorting by using digital sample enrichment and reverse transcription competitive allele-specific TaqMan PCR (dRT-castPCR) for rare mutations and qRT-PCR for cancer-specific genes. CastPCR is capable of detecting 1 mutant in 1 million wild-type molecules. Blood samples from lung cancer patients or normal individuals with spiked-in known lung cancer cell lines were partitioned in aliquots of 5 - 50 µL onto 96-well plates, such that each well contained either one cancer cell or none in the presence of 50 – 500 thousand normal white blood cells. Genetic mutations and panel of cancer-specific markers including CK19 and CEA for CTC identification and enumeration were determined by using both dCastPCR and qRT-PCR assays. **Results:** The sample partition process resulted in a digital enrichment of 20- to 200-fold (the relative ratio of CTC to normal cells) in a CTC-positive well. Digital castPCR accurately identified known mutation and CK19 in spiked-in samples of ~10 - 60 cells per mL whole blood by two cell lines, but there was no positive well in the absence of spiked-in cells. Furthermore, cell type specific markers (CK19) and known *EGFR* mutations were identified in the same sample wells, indicating that identified mutation was specifically derived from cancer cells. In five blood samples from lung cancer patients of stage I - IV, *EGFR* mutation (p.L858R) was detected in all samples. CTC numbers in 3 early- stage lung cancers (I and II) were 11 - 32 cells/mL blood. Much higher CTCs were detected in stage IV patients (>96 CTCs). For those sample wells with negative detection of *EGFR* mutation, corresponding wild-type sequences were detected in all sample wells. **Conclusions:** In conclusion, our data suggest that combination of digital sample enrichment with d-castPCR and qRT-PCR could be used to directly enumerate CTCs and detect cancer mutations in whole blood cells in early lung cancer patients without prior biophysical sample enrichment.

TT04. Detection of Clonality Using the InVivoScribe Technologies Assays for *IGH*, *TCRB*, and *TCRG* Gene Rearrangements by Capillary Electrophoresis on the Applied Biosystems® 3500

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Introduction: Gene rearrangement studies have been routinely used in the detection of clonal populations of B and T cells for the confirmation of diagnosis and detection of minimal residual disease. Many laboratories utilize capillary electrophoresis systems as the means for post-PCR detection of amplicons with the added ability of being able to accurately size these products. Here, we describe the use of the InVivoscribe Clonality kits on the new Applied Biosystems 3500. **Methods:** In the present study, we analyzed 10 specimens for *IGH* rearrangements and 6 specimens for *TCRB* and *TCRG* rearrangements. For *IGH* the specimens included 6 formalin-fixed, paraffin-embedded (FFPE) tissue samples (4 GI biopsies, 1 lymph node, 1 bone marrow (BM)), 1 BM aspirate, 2 peripheral bloods and 1 frozen BM tissue. For TCR studies the specimens included 1 BM aspirate, 1 peripheral blood and 4 FFPE skin biopsies. DNA was extracted using established protocols. After PCR amplification, capillary electrophoresis was performed on the AB 3500 at 60ºC using the POP-7™ polymer. The data were analyzed using GeneMapper 4.1 software. The reportable range was determined by analyzing a dilution series of positive controls for each primer set in concentrations of 200 ng, 100 ng, 50 ng and 10 ng total DNA template. Analytical sensitivity was determined using InVivoscribe sensitivity panels. Precision and accuracy were also evaluated. All specimens had previously been evaluated using the Beckman-Coulter Videra NsD. **Results:** In comparison with the Videra, 9/10 samples were concordant for *IGH* and 5/6 for TCR with the AB 3500. The *IGH* discordant case showed a clonal peak in FR2 with the AB 3500 (polyclonal in FR1 and FR3) that was not detected with the Videra. The discordant TCR case showed a clonal peak in tube TGA that was polyclonal on the Videra. The majority of samples were run in duplicate at 50 and 200 ng/µl concentrations and on multiple runs/days with the same results. For all *IGH* and TCR primer sets, all positive control concentrations (200ng to 10ng) were detected and with the sensitivity panels, all dilutions (100% to 1%) were detected. **Conclusions:** We have shown that the B cell *IGH* and T cell *TCRB* and *TCRG* InVivoscribe clonality kits can be used with excellent results on the AB 3500. The AB 3500 is able to detect clonal gene rearrangements with small amounts of DNA (10 ng/µl) with an analytical sensitivity of 1% in a variety of specimen sample types.

TT05. Comparison of the Performance of the Qiagen *BRAF* RGQ PCR and Autogenomics INFINITI® *BRAF* Assays Using DNA Extracted from FFPE Lung Tumor Biopsies

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Introduction: Mutations in the *BRAF* oncogene are common in melanomas and papillary thyroid cancers (PTC) and also found in other cancers of the lung and colon. The most common mutations, at codon 600, can result in constitutive activation of the *EGFR*-activated protein kinase signaling pathway. Testing for such mutations may be useful for staging patients with PTC and possibly melanoma, and for determining candidacy for treatment of specific cancers using monoclonal antibodies targeting the epidermal growth factor receptor. The purpose of this study was to evaluate two different commercial methods for detecting mutations in codon 600 of the *BRAF* gene. **Methods:** DNA was extracted from 10 µm FFPE lung tissue sections prepared from non-small-cell lung cancer (NSCLC) tumors using the Qiagen QIAamp DNA FFPE Tissue Kit. *BRAF* testing was performed using both the Qiagen *BRAF* RGQ PCR (real-time scorpion probe PCR) and the AutoGenomics (AGI) Infiniti *BRAF* (DNA microarray chip) assays, according to the manufacturer's instructions. The Qiagen method detects the V600E, V600D, and V600K mutations but does not differentiate which is being detected. The AGI method detects the V600A, V600E, V600D, and V600KRM mutations, and reports the specific mutation found. Real-time PCR for the Qiagen method was performed using the RotorGene Q platform (RGQ). Initial PCR amplification for the AGI test, and final mutation analysis, were performed with the Applied Biosystems 9700 thermocycler, and the AGI Infiniti Analyzer respectively. **Results:** Tissue sections from 25 NSCLC tumors, with known *BRAF* V600E mutation status, were analyzed by the Qiagen and AGI tests. *BRAF* testing results were in agreement for 24 of the 25 samples with both methods. One sample, positive for V600E with the Qiagen scorpion probe method, was wild type (WT) by the AGI microarray. This sample had been previously reported as WT by the laboratory that supplied the tissue sections, using Applied Biosystems SNaPshot® methodology. The Ct value of 38 for the discordant sample with the Qiagen assay was close to the cutoff of 40, suggesting that the percentage of cells carrying the mutation in the tumor may have been very low. **Conclusions:** Both *BRAF* mutation analysis methods tested are suitable for use in the clinical laboratory. The Qiagen and AGI methods have testing capacities of 30 and 22 samples per run, respectively, including controls. The advantage of the Qiagen *BRAF* test is rapid time-to-result, while that of the AGI method is greater coverage of codon 600 mutations, and reporting which mutation is detected.

TT06. Quality of DNA from Patients at Autopsy Varies with Collection Time and by Organ

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Introduction: The authors have embarked on a study to investigate potential differences in genomic copy number variations between organs from a single patient. We collected several tissue samples from patients at autopsy. The quality of the extracted DNA and its suitability for microarray analysis is an important issue for our study and allows us to investigate the wider issue of the quality and suitability of all samples extracted at autopsy, which is a fairly common practice. Much attention has been paid to the quality of nucleic acid derived from formalin-fixed, paraffin-embedded tissues, but few, if any, articles discuss the quality of DNA from fresh autopsy-derived tissue, particularly between organs from a single individual. **Methods:** Five patients

who died within 48h of autopsy examination and for whom the families could give consent, were selected for study. All patients died in hospital and were transferred to a cool (5˚C) room within 3 hours of death. Ten organs plus peripheral blood from each patient were sampled: skin, cardiac muscle, skeletal muscle, lung, colon, stomach, spleen, pancreas, kidney, and brain. Solid tissue samples were collected without media, peripheral blood was collected in PAX DNA tubes; all samples were frozen within one hour of collection. DNA was isolated using Qiagen kits, and quality was assessed by agarose gel electophoresis. The patients' hospital charts were reviewed for demographic data as well as past medical history, history of presenting illness and cause of death. **Results:** The DNA quality was highly variable between organs and patients. Representative gel images of genomic DNA from each organ and each patient will be presented. The interval between time of death and time of autopsy examination varied from 7 to 45 hours; longer intervals correspond to poorer DNA quality. The variability between organs was not consistent between patients, but spleen, stomach and pancreas-derived DNA were generally of the worst quality. Skin and skeletal muscle samples were generally the best. **Conclusions:** The finding that DNA quality may be negatively correlated with the time between death and autopsy is consistent with published data, and detailed examination of a larger number of subjects may allow us to develop guidelines for tissue sampling. The variability in DNA quality between organs from the same individual is interesting and suggests that some tissues may be superior for collection when DNA studies or banking are required. We intend to attempt to correlate the internal variability with each patient's medical history.

TT07. Rapid Identification of Somatic Mutations in Melanoma Cell Lines

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Introduction: Signaling pathways, including *MAPK* and *KIT*, are known to regulate cell proliferation and intracellular processes in multiple cancer types, including malignant melanoma. Malignant melanomas are known to have activating mutations within several of these pathways that may serve as potential therapeutic targets yet making the identification of multiple mutations a financial and time limiting step. In this study, we evaluated a method for rapidly identifying somatic mutations of interest in melanoma cell lines. **Methods:** Eleven experimental and one control cell lines were grown in culture until log phase had been reached. Cells were then harvested and DNA was extracted using the Qiagen QIAamp DNA Mini Kit. Following DNA extraction, qPCR of each cell line was performed using the Qiagen qBiomarker™ Somatic Mutation PCR Array, directed at the Human *KIT* pathway, and run on the AB 7500 Fast Real-time PCR System. The array includes mutational analysis of the *AKT, BRAF*, *EGFR*, *KRAS*, *HRAS*, *NRAS*, *MEK1*, *PIK3CA*, and *PTEN* genes. The mutational status of each experimental cell line was determined using the $\Delta\Delta$Ct method. The control cell line (human fibroblasts) was run on the array in triplicate, and the ΔCt for each run averaged for use in the analysis. **Results:** Eleven experimental cell lines were examined for somatic mutations of genes within the human KIT pathway. Three of the cell lines (HMCB, WC00062 and WC00080) examined were wild type for the 85 mutations tested. Eight (72%) cell lines were shown to have a mutation in the *BRAF* (V600E) gene, and of these eight, three showed additional mutations. In the cell lines with multiple mutations, HS294T and VMM12 harbored a mutation in the *KIT* (M541L) gene while WCOO46 harbored two unique mutations in the *KIT* gene (M541L and L576E). **Conclusions:** Identifying somatic mutation in cell lines used in *in vitro* experiments is an important step in identifying potential therapeutic targets of malignant melanoma. Here we identified somatic mutations in eight cell lines of interest. Using the qBiomarker™ Somatic Mutation PCR Arrays allowed us to rapidly identify these mutations with limited hands on time required and at a reduced cost compared to traditional SNP genotyping methods.

TT08. Taking Ultra-Deep Sequencing to the Extreme: COLD-PCR Transforms the Mutation-Detection Capability of Next Generation, Targeted Amplicon Resequencing

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Introduction: There is tremendous interest in the application of next generation sequencing (NGS) for the analysis of individual cancer specimens for personalized treatment-guidance, prognosis, and therapy follow-up. A major obstacle with many classes of clinical specimens is that tumor cells are mixed with normal/stromal cells; thus the resulting low-level DNA mutations fall below the current NGS detection limit (i.e., false positives predominate in such samples). Because low-level tumor mutations can have profound implications on the development of metastasis, prognosis, choice of treatment, follow-up or early detection strategies, it is imperative to ensure their detection. **Methods:** Herein, we amplify the genomic DNA of serially-diluted mutation-containing human cell-lines into wild-type DNA, as well as clinical tumor specimens, using COLD-PCR (a PCR technology that enables enrichment of unknown mutations at any sequence position), as well as conventional PCR for comparison. Following the amplification of several *TP53*, *KRAS* and *EGFR* regions with mutations, PCR products were pooled and multiplexed through library preparation, and we analyzed the mutation detection sensitivity of targeted amplicon resequencing on the Illumina HiSeq2000 next generation sequencing platform. **Results:** Regardless of sequence depth, we observed a mutation-detection limit of approximately 1% to 2% mutation abundance in conventional PCR amplicons; in contrast, COLD-PCR amplicons encompassed mutation enrichment exceeding the sequence noise levels, and enabled ultra-deep identification of mutant abundances of as little as 0.02% in wild-type DNA. Through the analysis of clinical tumor specimens, we have demonstrated that several low-abundance mutations cannot be detected in next generation sequencing of conventional amplicons, yet are clearly detectable in the COLD-PCR amplicons. **Conclusions:** We have observed that increased depth of sequence analysis does not overcome background noise using Illumina next generation sequencing, and thus does not allow for the detection of mutation abundances below ~1% to 2% in clinical specimens. However, combining COLD-PCR with amplicon resequencing magnifies the mutations and enables reliable NGS sequencing of mutation spectra. It is anticipated that COLD-PCR will facilitate the reliable application of NGS for many clinical specimens such as: infiltrating, diffuse-type tumor specimens; sub-optimally micro-dissected or heterogeneous tumor samples; DNA from circulating nucleic acid, circulating cells, sputum or other bodily fluids; tumor margins; stromal cells; and generally facilitate the broad inter-phasing of NGS with clinical practice.

TT09. Validation of Cytochrome p450 2D6 Genotyping by the Luminex xTAG® 2D6 v3 IVD/LX200 Detection System

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Introduction: The cytochrome p450 2D6 enzyme is important for metabolism of many exogenous drugs in clinical use, including activation of the breast cancer hormone modulator tamoxifen. The *CYP2D6* gene exhibits significant inter-individual variability, which is associated with altered response to medications metabolized by *CYP2D6*. We evaluated *CYP2D6* genotyping using the Luminex xTAG® *CYP2D6* Kit v3, which detects 17 different *CYP2D6* alleles including gene duplication, deletion, frameshift mutations, and point mutations that result in increased, decreased, or no enzymatic activity. **Methods:** A total of 46 DNA specimens containing a variety of *CYP2D6* allele combinations were used for validation of the Luminex xTAG® *CYP2D6* genotyping method. The samples included 20 DNA samples from a previous clinical study, two samples from the Coriell cell repository, two samples from LabCorp, and six samples from Luminex. In addition, 16 randomly selected whole blood specimens were tested to validate use with different DNA extraction methods. Genotyping was performed as per manufacturer instructions with an input of approximately 50 ng of genomic DNA. Results were compared to prior genotyping results from the Roche AmpliChip *CYP450* test or the Coriell database. Samples with discrepant results were further analyzed by DNA sequencing in order to identify the correct genotypes. **Results:** Concordance for *CYP2D6* genotype was obtained for 24 of the 30 comparison samples. Discrepancies were observed for six of the validation samples. Four samples were homozygous for *CYP2D6*17* (*17/*17) on the Roche AmpliChip, but were called *CYP2D6*17* heterozygous (*2/*17) by Luminex. Two samples were compound heterozygous for a gene deletion and *CYP2D6 *17* (**5/*17*) on the AmpliChip but received no call by Luminex. Further analysis by DNA sequencing using primers flanking *CYP2D6* exon 2 confirmed homozygous or hemizygous presence of the *17 variant nucleotide 1023C>T for all six of the discrepant samples, confirming previous genotyping results. Analysis of the raw Luminex data revealed a high signal with the *17 wild-type probe for each of these samples, resulting in either a no-call or *17 heterozygous miscall. All 16 in-house extracted DNA samples were successfully genotyped. **Conclusions:** The Luminex xTAG® *CYP2D6* v3 genotyping method was able to identify 25 different allele combinations of *CYP2D6*. Correct genotyping calls were made for all alleles analyzed with the exception of samples homozygous or hemizygous for the *CYP2D6 *17* 1023C>T variant. The Luminex method was robust and easy to use with a short turnaround time and reasonable hands-on time.

TT10. COLD-PCR in Emulsion Magnifies Low-Level Mutations Prior to Sequencing

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Introduction: Reliable sequencing of low-level mutations in certain classes of clinical samples (infiltrating, diffuse-type tumor specimens; tumor-margins; stromal cells; DNA from circulating-nucleic acids, circulating-cells, sputum or other bodily samples) is of

paramount significance for personalized medicine based upon mutational profiling of individual patients. Nevertheless, Next-Generation-Sequencing (NGS) technologies are not sufficiently reliable for the discovery or validation of low-level mutations in clinical samples, and thus NGS integration within Clinical Molecular Diagnostics cannot be exploited effectively. Employing COLD-PCR, a technology that magnifies mutations at any sequence position of a given amplicon, enables enrichment of low-level mutations such that NGS technologies can easily identify them (see Abstract TT08 by Milbury et al, AMP 2011). Despite this advantage, COLD-PCR requires individualized optimization for each DNA target amplicon, and thus COLD-PCR is laborious and technically challenging for large-scale analyses. We examined the feasibility of simultaneously performing COLD-PCR in several sequences, by adapting COLD-PCR to operate in nano-PCR emulsion-based reactions in the presence of several copies of a distinct DNA target sequence. Accumulation of the target sequence within individual emulsions was enabled by creating magnetic beads coated with specially designed Reference Sequences (COLD-PCR-beads). **Methods:** COLD-PCR-beads were prepared using streptavidin-coated beads possessing several copies of a single-stranded Reference Sequence. The COLD-PCR-beads capture numerous copies (both mutant and wild type) of target sequences from a diverse DNA population. Genomic DNA from human cell-lines that harbor *TP53* mutations (c.847C>T, p.R283C and c.818G>A, p. R273H respectively), were serially-diluted into wild-type DNA for subsequent experiments. Single-stranded target DNA, generated with an asymmetric PCR approach, was used for magnetic hybridization capture (MHC) on COLD-PCR beads. Following the capture of target sequences, COLD-PCR-beads were emulsified such that each emulsion contains at most one bead. Fast-COLD-PCR was performed at a denaturation temperature determined experimentally. **Results:** COLD-PCR performed within emulsion (eCOLD-PCR) resulted in the enrichment of mutations in all three sequences examined. Mutations located in different regions of *TP53* exon 8 showed an average 7-fold mutation enrichment during eCOLD-PCR. A second round of MHC and eCOLD-PCR produced additional 5-fold mutation enrichment. eCOLD-PCR produces similar mutation enrichment to that produced in-solution and following eCOLD-PCR DNA target amplicons with mutations at the 1% level or below could be directly sequenced via Sanger-sequencing. **Conclusions:** eCOLD-PCR enables miniaturization of COLD-PCR and opens up the possibility for highly-parallel COLD-PCR reactions in several target sequences simultaneously, followed by reliable NGS-based detection of low-level mutations. eCOLD-PCR thus may facilitate broad inter-phasing of NGS with Clinical Diagnostics.

TT11. A Milling Machine Based Meso Level Dissection Device for Accurate, Rapid, and Economical Recovery of Tissue from Slides

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Introduction: Dissection of formalin-fixed, paraffin-embedded (FFPE) sections of solid tumors is required for most molecular oncology testing, since the analytical sensitivity depends upon the purity of tumor tissue obtained. Most pathology laboratories employ manual dissection due to the high cost and labor intensiveness of currently available microdissection instrumentation, such as laser capture microdissection (LCM) instruments. We have developed an inexpensive milling machine based mesodissection prototype instrument that provides an intermediate level of resolution. This mesodissection instrument employs a specialized milling tip that simultaneously dispenses and aspirates milling liquid in order to capture the milled fragments. This instrument is fast and easy to use, capable of transferring electronically indicated target regions between neighboring tissue sections, and leaves intact non-dissected tissue allowing improved documentation of the region that was sampled. This is the first report of the performance of this prototype mesodissection instrument. **Methods:** Performance criteria were developed for resolution, accuracy, purity, and efficiency. Resolution and accuracy were assessed by microscopic inspection of the dissected region; resolution is the smallest recoverable area and accuracy is the dissected region in comparison to the boundaries of the desired region. Purity is defined as the percentage of desired tissue retrieved from a mouse/human fusion FFPE block as determined by real-time PCR amplification of species-specific repetitive DNA elements. Efficiency is defined as total DNA recovered relative to manual methods, as quantified by PicoGreen fluorescence. **Results:** Currently, the working prototype is capable of 0.5 mm resolution, accuracy better than 0.1 mm compared to a desired boundary, and efficiency comparable to manual methods. Improvements to the prototype and evaluation of performance criteria are currently ongoing. **Conclusions:** The mesodissection instrument provides an alternative to the limited resolution of manual dissection methods and the high cost and low recovery efficiency of LCM instrumentation for dissection of selected areas from FFPE specimens mounted on slides. Digitally targeted dissection using adjacent H&E-stained tissue sections marked by a pathologist allows for downstream dissection by a technologist without specialized training in histopathology.

TT12. A Quantitative Real-Time PCR Based Approach for Resolution of *HER2* Amplification/Over-Expression Status in FISH and IHC "Double Equivocal" Breast Cancer

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Introduction: This study assesses the feasibility of using Quantitative Real-Time PCR (qRT-PCR) to resolve *HER2* amplification/over-expression status in invasive breast cancer cases that fail resolution via immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) testing following ASCO/CAP guidelines. The IHC and FISH equivocal patient population (Double Equivocal) represent a particularly problematic breast cancer sub-group that currently lacks standardized clinical management guidelines. **Methods:** Cases were selected from the Cleveland Clinic electronic records from 1/2008 to 12/2010. RNA extraction was performed following macro-dissection using High Pure RNA Paraffin Kit (Roche Applied Biosciences, Indianapolis, IN). qRT-PCR was carried out using TaqMan® RNA-to-CT™ 1-Step Kit with primers and probes (*HER2*, *B2M*, *GAPDH*, *ACTB*, *TFRC*, Applied Biosystems, Foster City, CA). qRT-PCR was performed on a LightCycler 480 II (Roche Applied Biosciences, Penzberg, Germany) according to the manufacturer's instructions. Results were expressed as the ratio of *HER2* to reference gene copies, all normalized against calibrator RNA from MCF7. **Results: q**RT-PCR performed on two breast cancer control groups, *HER2* amplified (AMP) and non-amplified (Non-AMP) as defined by FISH and IHC, demonstrated 2 non-overlapping populations. ROC curve analysis, using a cut off of 7.0, showed the qRT-PCR assay separates AMP from Non-AMP cases with 100% sensitivity and specificity. Applying the 7.0 RT-PCR cut off to a group of double equivocal cases resulted in resolution of *HER2* amplification/expression status for all cases (10 AMP and 40 Non-AMP). Cases with heterogeneity of *HER2* expression did not alter sensitivity of the RT-PCR assay. **Conclusions: q**RT-PCR analysis of *HER2* gene expression represents a viable approach to resolve cases with double equivocal *HER2* status at the time of diagnostic biopsy. This molecular approach accurately determined *HER2* status in a population that failed classification by both FISH and IHC. qRT-PCR combines the precision and high sensitivity of real-time PCR with the morphological specificity of histological evaluation and ultimately allows definitive *HER2* classification at the time of initial diagnosis. Further studies correlating response to anti-*HER2* therapy and *HER2* status by qRT-PCR are warranted.

TT13. Improvement of Pyrosequencing Assay for *EGFR* Exon 19 Deletions

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Introduction: *EGFR* exon 19 mutation has different variants, which pose a challenge for pyrosequencing. To increase the capacity of the assay in detecting exon 19 mutations, a restriction enzyme (MseI) digestion has been used to enrich mutant *EGFR* exon 19 in specimen. There are MseI restriction sites in *EGFR* exon 19. Most of exon 19 deletions will eliminate one MseI restriction site. Therefore, the *EGFR* exon 19 deletion mutant is more resistant to MseI digestion. After MseI digestion, the ratio of exon 19 deletion mutant in a specimen will be increased and easier to be detected and easier to be analyzed. **Methods:** The DNA from an *EGFR* exon 19 deletion mutant cell line PC9 was mixed with normal spleen cell DNA at different ratios to generate 5%, 2.5%, 1.25%, 0.625% and 0.3125% preparations. A portion of the above preparations was subjected to MseI digestion before and after PCR amplification. (10 unit/10 µl, 37 degrees for 3 hours). The rest is served as control. The preparations with or without digestion were pyrosequenced. Five consecutive *EGFR* exon 19 deletion positive samples were selected and de-identified from routine molecular test specimen. The sequencing results of these samples with or without digestion were also compared. Pyrosequencing reagents were purchased from Qiagen. Pyromark MD was used according to the manufacturer's instructions. **Results:** Without MseI digestion, the exon 19 mutation was detected in 5%, 2.5%, 1.25% samples, but not in 0.625% and 0.3125% samples with quantitating results of 4.97%, 2.72%, 2.20%, 0%, 0%, respectively (see figure 2A). With MseI digestion, the exon 19 mutant was detected in all 5 preparations (5%, 2.5%, 1.25%, 0.625% and 0.3125%) with quantitating results of 85.43%, 81.54%, 75.06%, 62.72% and 58.78%respectively. FFPE tissue was also tested with or without MseI digestion. After MseI digestion, the peak height from mutant increased. The negative cases were also tested. After digestion, the negative cases remained negative. **Conclusions:** MseI digestion could increase pyrosequencing assay sensitivity for *EGFR* exon 19 deletion approximately 20 to 200-fold. The enrichment is more effective when starting mutant gene percentage is low. The digestion also simplifies the data analysis and also works in FFPE specimen.

TT14. Multiplex Sequencing: Using Multiplex PCR Amplification and Pyrosequencing for *KRAS*/*BRAF* Mutation Analysis

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Introduction: Novel targeted therapeutic approaches often require detection of a specific mutated gene in a patient's tumor. In addition, mutations in downstream effectors of drug targets can impact therapeutic efficacy. Targeted agents to the epidermal growth factor receptor (*EGFR*) have improved outcomes for patients with metastatic colorectal carcinomas (mCRCs). However, activating mutations in the *KRAS* or *BRAF* genes confer resistance to anti-*EGFR* agents. *KRAS* mutation status of mCRC cases is now routine, but *BRAF* gene status is only recommended as an option if *KRAS* is wild type. In addition, mutation in the *KRAS* codon 61, although much less frequent, also confers resistance. Although PCR can be multiplexed to detect the most frequent mutations, sequencing is the method of choice to detect all possible mutations but needs to be done individually for each exon. Our goal was to develop a multiplexed sequencing assay that could be applied to *KRAS*/*BRAF* mutation detection. **Methods:** We designed a multiplex PCR protocol to amplify gene sequences containing *KRAS* codons 12/13, *KRAS* codon 61, and *BRAF* codon 600. Pyrosequencing was performed using the multiplex amplicon reaction. A *KRAS* (codon 13) mutant cell line (HCT116), a *BRAF* (codon 600) mutant cell line (HT29), and a wild-type control (placental DNA) were tested simultaneously. We also determined the lower limit of detection of *KRAS* and *BRAF* mutations. **Results:** The multiplex PCR yielded specific amplicons for each of the target sequences. The locus containing *KRAS* codon 61 was more efficiently amplified and appeared to negatively influence the yield of product from the less efficient locus containing *KRAS* codon 12/13. Pyrosequencing demonstrated expected sequencing patterns (i.e., mutated sequences c.38G>A (p.Gly13Asp) and c.1799T>A (p.Val600Glu) in *KRAS* and *BRAF* mutant cell lines, respectively, and wild-type sequences in control DNAs). However, there was slightly increased background noise compared to non-multiplex PCR reactions. The lower limit of detection was 8% (allele burden) for *KRAS* G13D mutation and 14% (allele burden) for *BRAF* V600E mutation. **Conclusions:** We have demonstrated that the clinically significant exons of *KRAS* and *BRAF* in CRCs can be tested simultaneously with our multiplex PCR reaction protocol. This approach could enable a single multiplex PCR reaction followed by sequential sequencing for multiple mutations (proceeding only to the next step if wild type) in other signaling pathways. Further optimization and validation with a large number of samples will be necessary for clinical implementation of our *KRAS*/*BRAF* assay.

TT15. Automated DNA Extraction from FFPE Tissue Using a Xylene-Free Deparaffinization Method

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Introduction: Formalin-fixed paraffin-embedded (FFPE) tissue samples are routinely used for immunohistochemistry and molecular analysis in cancer research. However, many methods for DNA extraction from FFPE tissue sections are manual procedures that are not standardized, time consuming and often include usage of hazardous materials like xylene. In this study, we describe an alternative xylene-free deparaffinization method that can be used in combination with automated DNA extraction on QIAsymphony sample preparation instrument. Performance is shown in comparison to DNA extraction using standard xylene deparaffinization method and manual sample preparation. **Methods:** Either rat FFPE tissue material was prepared freshly or human FFPE Tissue was purchased from Proteogenex. Up to eight tissue sections were used for DNA extraction. Deparaffinization of FFPE tissue was performed according to QIAamp DNA FFPE Tissue Kit by use of xylene and ethanol or by use of QIAGEN Deparaffinization solution. DNA was extracted by either using QIAsymphony DNA Mini Kit in combination with the Tissue Low content protocol or manual QIAamp DNA FFPE Tissue kit. Performance was compared by determining DNA yield, purity and integrity as well as mutational status of biomarker. Linearity of DNA extraction was assayed by using increasing amounts of FFPE tissue sections. **Results:** Hands-on-time for deparaffinization was significantly reduced from 20min to 5min by use of Deparaffinization solution. Data obtained by performance study using xylene-free deparaffinization showed a mean DNA yield that was comparable to standard xylene pre-treatment or manual method. DNA purity determined by the ratio A260nm/280nm was greater than 1.8 for all methods. Integrity of DNA was tested by multiplex endpoint PCR that showed the same spectrum of amplicon sizes and demonstrated the usability of DNA for PCR analysis. A plot of DNA yield vs. amount of FFPE sections revealed linearity for automated methods (regression coefficient of ≥ 0.9). Analysis of mutational status of biomarker *KRAS*, *BRAF* and *EGFR* lead to identical results, demonstrating functionality of extracted DNA. **Conclusions:** The QIAGEN Deparaffinization solution in combination with QIAsymphony DNA Mini Kit and the Tissue Low content protocol represents an efficient and fast method for automated DNA extraction from FFPE tissue without use of the hazardous material xylene.

The applications presented here are for research purposes. Not for use in diagnostic procedures.

TT16. Fluorescence-Based Multiplex PCR MSI Assay Optimized for the Applied Biosystems 3500 Genetic Analyzer

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Introduction: Approximately 15% of colon cancers demonstrate high frequency microsatellite instability (MSI-H). As MSI-H patients have better overall survival compared to patients with microsatellite stable (MSS) colon cancer and do not respond as well to 5-fluorouracil-based adjuvant chemotherapy, identification of MSI-H colon cancer is clinically significant. MSI status is frequently determined by multiplex PCR of five mononucleotide alleles followed capillary gel electrophoresis. We describe a modified PCR protocol for MSI detection that is well-suited for the Applied Biosystems 3500 Genetic Analyzer, which recently became available in the USA. **Methods:** 40 paired normal and tumor DNA samples were purified from FFPE colon cancers. 0.4 to 26.8 ng of template DNA was used to analyze NR27, NR21, NR24, BAT25, and BAT26 mononucleotide microsatellite loci in multiplex PCR. Reverse PCR primers were labeled with 6FAM, VIC, or NED fluorescent dyes. The PCR products were resolved on an Applied Biosystems 3500 Genetic Analyzer and analyzed using GeneMapper 4.1 software. Cases were also analyzed by immunohistochemistry (*MLH1*, *MSH2*, *MSH6*, and *PMS2*). 20 of the cases were analyzed in parallel using a commercially available multiplex PCR MSI Analysis System. **Results:** Total PCR time was 75 minutes. Fluorescent intensities of all markers were balanced allowing maximum sensitivity. Shifted peaks from a DNA sample containing 5% of MSI DNA were clearly identified. The minimum detection limit of template DNA was 0.4 ng. Robust PCR amplification of all mononucleotide loci in all samples was observed, and all results were confirmed by IHC. In marked contrast to our results, weak amplification of one or more loci was observed in 4 samples tested using conventional multiplex PCR methods in conjunction with an older-model capillary gel electrophoresis instrument. Consequently the MSS status of these cases could not be unambiguously established. **Conclusions:** The modified MSI assay described here is optimized for the Applied Biosystems 3500 Genetic Analyzer. The assay is fast, robust, relatively inexpensive, and sensitive down to less than 0.5ng of purified template DNA. To the best of our knowledge, no MSI detection kits are commercially available for the Applied Biosystems 3500 Genetic Analyzer. When compared with an existing MSI Analysis System optimized for older model capillary gel electrophesis instruments, this modified MSI assay generates more intense peaks making subsequent interpretation less challenging while also reducing the turn-around-time for MSI results.

TT17. Performance Evaluation and Comparative Simulation Modeling of LightCycler® MRSA Advanced and Xpert™ MRSA assay in a Multi-Center Trial

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Introduction: Rapid detection of nasal colonization with methicillin-resistant *Staphylococcus aureus* (MRSA) followed by infection control procedures can curtail MRSA infection and transmission. Universal screening imposes considerable demands on performing laboratories and optimization is key to achieving acceptable turnaround times. When tests have comparable sensitivity and specificity, the selection of one technology over other is usually based on determining laboratory efficiency parameters such as throughput, turnaround times, labor utilization and cost, which is best determined by simulation modeling. **Methods:** The performance and work-flow of two FDA approved tests, the LightCycler® MRSA Advanced test (Roche Indianapolis, IN) and Xpert™ MRSA test (Cepheid Sunnyvale, CA) was evaluated on 1202 specimens in a multi-center trial. It was noted that the mean hands on time for sample preparation (using all samples regardless of whether they had been tested in singlicate or in groups/batches) was actually less for the LightCycler® MRSA Advanced assay than the Xpert™ MRSA assay per sample (1:40 min vs 2:35 min). We used a discrete event simulator to simulate the processes and confirm without bias using iGrafx Process 2007 for Six Sigma discrete event simulator software. Time and motion analysis was performed for all steps in the two processes. The models were validated by comparing the predicted turnaround times and test throughput with the experimental data. **Results:** Compared to directly-plated culture, the LightCycler® MRSA Advanced and Xpert™ MRSA assay showed similar relative sensitivities of 95.2 (95% CI 89.1-98.4%) and 99.0% (95% CI 94.8-100%), and specificities of 95.5 (94.1-96.7%) and 95.5% (95% CI 94.1-96.7%) respectively. The GeneXpert system is well suited when single samples are tested giving better turnaround times (73 min as compared to 112 min) as long as

number of samples tested per run is less than or equal to 8. However, when MRSA is tested in batch mode (n= 30) as was done at two of the sites, the LightCycler gives better throughput, shorter turnaround times (213 min compared to 286 min) and comparable percent labor utilization (17.9% and 16.6%). **Conclusions:** The low labor utilization clearly indicates that the rate limiting factor for test throughput is the number of GeneXpert modules available in the laboratory. The multiple sample processing capabilities of the LightCycler® offers advantages for batch mode processing. Such optimization models are clearly valuable for successful laboratory operations.

TT18. Comparison of SNaPshot® Fluorescent Capillary Electrophoresis and Sequenom® MassARRAY Based Platforms for Multiplex Solid Tumor Mutation Screening

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Introduction: With increasing availability of targeted therapies for solid tumors, simultaneous assessment of the mutation status of multiple targetable genes in tumors has become critical for patient management. Limited availability of samples necessitates the use of multiplex approaches for testing. Current multiplex hotspot mutation screening approaches include primer extension assays followed by mass spectrometry (MS)-based (Sequenom® MassARRAY; Sequenom, San Diego, CA) or fluorescent capillary electrophoresis (FCE)-based (SNaPshot® multiplex system: Life Technologies, Carlsbad, CA) detections. As the FCE-based platform, which offers flexibility for multiple applications, is available in many molecular diagnostic laboratories, we validated a solid tumor mutation screening panel using this platform. Here, we present our assessment of the performance characteristics of the FCE-based mutation panel in comparison with our existing MS-based mutation panel. **Methods:** DNA was extracted from 7 formalin-fixed, paraffin-embedded (FFPE) cell lines (HL60, A375, H2122, H1437, HT1080, DLD1 and CAMA-1) and 120 manually micro-dissected FFPE solid tumor samples. A 9-well panel was developed to assess the mutation status of hotspot regions from 13 genes (*AKT 1*, *AKT 2, AKT 3, BRAF*, *EGFR*, *GNAQ*, *GNAS*, *IDH1*, *IDH2*, *KRAS*, *MET*, *PIK3CA*, and *RET*). DNA (10 ng) was PCR amplified and subjected in parallel to single-base-primer extension (SBE) using the SNaPshot® multiplex kit and iPlex® Pro kit (Sequenom). SBE products were genotyped by FCE-based or MS-based detection. Assay sensitivities were determined for mutations for which positive cell lines were available. **Results:** Both FCE-based and MS-based panels successfully detected all previously published mutations in the control cell lines. Sixty-seven mutations (*KRAS*, N=21; *NRAS*, N=4; *MET*, N=8; *PIK3CA*, N=19; *BRAF*, N=9; *IDH1*, N=2; *EGFR*, N=1; *GNAQ*, N=2; and *GNAS*, N=1) were detected in 57 tumor samples. One hundred percent concordance was observed in the genotyping results between the two platforms. Depending on the level of multiplexing in a given well, the sensitivity of detection ranged from 3% to 7% for FCE-based panel and from 5% to 10% for MS-based panel. **Conclusions:** Both FCE and MS provide reliable high-throughput platforms that can be used to detect multiple mutations simultaneously using limited DNA. In our experience, FCE data were easier to interpret, especially in samples with low level mutations, as the visualization was aided by both color and fragment size. Further, the FCE-based panel can be easily incorporated into the workflow of most molecular diagnostic laboratories by using existing genetic analyzers that are already in wide-spread use for fragment analysis and Sanger sequencing.

TT19. Verification of a Fully Automated Sample Preparation for SurePath™ Specimens Using the QIAsymphony SP

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Introduction: QIAGEN is currently developing a DNA extraction procedure based on pH-driven anion exchange sample preparation chemistry (AXpH) for use on the QIAsymphony SP. This work summarizes data generated using SurePath specimens on the QIAsymphony SP. **Methods:** To compare manual and automated sample preparation methods, DNA was isolated on the QIAsymphony using the AXpH SurePath protocol and as a reference, the manual sample conversion method. The QIAsymphony eluates and the corresponding manually converted pellets were tested with digene HC2 High-Risk HPV DNA Test®. HC2 testing was done using 352 residual, de-identified, cervical SurePath post-gradient specimens, retained after cytology screening. The automated DNA extraction procedure was evaluated for repeatability, precision, eluate stability and tested the effect of potentially interfering substances on the extraction procedure using either pools of de-identified SurePath samples, or individual cervical SurePath post-gradient specimens. To test repeatability, DNA was purified from 7 dilutions of HPV positive samples in two independent experiments using the QIAsymphony DSP AXpH DNA Kit. To test precision, inter-instrument and inter-day precision was determined on three different QIAsymphony instruments and on three different days using HPV positive samples. For eluate stability analysis, DNA extracted from positive and negative material was stored 4-8°C or at –20°C prior to analysis in HC2. The impact of potentially interfering substances on the AXpH chemistry were tested by adding varying amounts of blood, lubricating jelly, contraceptive jelly, spermicidal gel, douche, feminine spray, and antifungal cream to low positive samples. **Results:** The QIAsymphony AXpH SurePath protocol resulted in 92.9% total agreement with the manual conversion method (327 of the 352 results agreed, kappa = 0.81), negative agreement was 96.6% (255 of the 264 results agreed), and positive agreement was 81.8% (72 of the 88 results agreed). (b) Initial evaluation of the automated procedure showed that the specifications for repeatability, precision and the effect of potentially interfering substances were met. Preliminary eluate stability results for up to two weeks at 2–8°C and up to four weeks at –20°C met the acceptance criteria. **Conclusions:** We successfully evaluated the automated QIAsymphony DSP AXpH DNA extraction procedure using SurePath™ samples. The QIAsymphony applications presented here are for research purposes. Not for use in diagnostic procedures.

TT20. Implementation of *IL28B* rs12979860 Polymorphism Testing on the Abbott m2000 Platform

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Introduction: Genome wide association studies have identified the single nucleotide polymorphism (SNP) rs12979860 as an important predictor of Hepatitis C virus treatment response and spontaneous viral clearance. rs12979860 genotype results are becoming an important component of HCV therapeutic planning and management. We implemented a rapid, robust real-time TaqMan PCR assay to determine the rs12979860 genotype utilizing the Abbott m2000 platform. **Methods:** Genomic DNA was extracted from 50 de-identified EDTA whole blood samples utilizing the whole blood protocol on the Abbott m2000sp instrument. The extracted DNA was analyzed by real-time TaqMan PCR on the Abbott m2000rt real-time PCR System with hydrolysis probes specific for the rs12979860 C and T alleles. Results were correlated with genotypes obtained by Sanger sequencing and with results obtained from an outside reference laboratory. **Results:** Assay performance was excellent with all of the fifty samples generating an appropriate genotype result. There was 100% agreement with Sanger sequencing results and reference laboratory results. Allelic frequencies were 0.618 for the C allele and 0.382 for the T allele. Genotype frequencies were C/C 0.417, C/T 0.402, and T/T 0.181. The allelic and genotype frequencies observed were similar to published frequencies, however the ethnic background of the patients tested was not available and the tested population likely represents a mixed ethnicity population. The genotype results were highly reproducible between runs, with repeat analysis (n=9) showing a CV of 1.5% for the Ct value of the C allele and CV of 0.2% for the Ct value of the T allele. **Conclusions:** Our implementation of a rs12979860 genotyping assay utilizing the Abbott m2000 platform is readily applicable to clinical testing and demonstrates excellent performance and reproducibility. Rapid determination of the rs12979860 genotype will facilitate the planning and management of personalized treatment for HCV infected individuals.

TT21. A Comparative Study of Methods for Determination of *NPM1* Mutation Status in Diagnostic AML Specimens

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Introduction: Mutations in nucleophosmin (*NPM1*) are important for stratification of patients with cytogenetically normal acute myeloid leukemia (AML) with respect to the timing and use of induction chemotherapy and stem cell transplant. Mutations in *NPM1* are most commonly the result of a 4 bp insertion at a specific site in exon 12. Numerous mutations have been described; however, most mutation positive patients have one of three insertion mutations (types A, B, D). Several methods have been proposed for detection of *NPM1* mutations in patient samples, including sequencing and liquid bead array based methods, each with inherent advantages and disadvantages with respect to analytical and clinical sensitivity and ease of interpretation. **Methods:** Diagnostic AML specimens (bone marrow and peripheral blood, N=64) from patients at the Hospital of the University of Pennsylvania were collected, and RNA and DNA were extracted using the QIAamp RNA Blood mini kit and QIAamp DNA blood mini kit (Qiagen, Valencia, CA), respectively. Each specimen was evaluated by Sanger sequencing (BigDye Terminator method, Applied Biosystems, Carlsbad, CA), pyrosequencing (Pyromark, Qiagen) and a liquid bead array assay (Signature *NPM1* Research Use Only Kit, Asuragen Inc., Austin, TX) to assess *NPM1* mutation status. Clinicopathologic data were also obtained for correlation with *NPM1* mutation status. **Results:** *NPM1* mutations were detected in 20/64 specimens tested (31%). Forty-two of the patients had cytogenetically normal AML, and *NPM1* mutations were detected in 17 of these patients (40%). These results are comparable to those previously reported.

Overall concordance between the methods was excellent. Data on the performance of each method will be presented including 4 samples that upon sequencing presented interpretive challenges. Notably, the liquid bead array assay demonstrated commendable performance characteristics including 100% concordance with sequencing as well as an analytical sensitivity at least as low as 0.01% as determined by an admixture of OCL-AML3 cells and HL-60 cells. **Conclusions:** While multiple methods are available for the determination of *NPM1* mutation status, the Signature *NPM1* liquid bead array assay is a sensitive and straightforward method to asses for *NPM1* mutations in diagnostic AML patient samples. This is in contrast to the potentially difficult analysis of data generated by less sensitive methods such as traditional Sanger sequencing and pyrosequencing. Although the liquid bead array assay cannot distinguish amongst the *NPM1* mutations it is designed to detect, more than 90% of known *NPM1* mutations are covered by the assay.

TT22. Development and Validation of SNP Detection Assays Using Invader Plus® Reagents

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Introduction: Single Nucleotide Polymorphisms (SNPs) are often linked to the development of diseases such as cancer and resistance/sensitivity to specific chemotherapeutics. Detection of such gene alterations is fundamental in the diagnosis of cancer or in treatment management. We have designed, developed and validated two assays for the detection of *BRAF* V600E and DPD IVS14+1G>A. The *BRAF* V600E mutation may be associated with resistance to anti-*EGFR* drugs such as panitumumab and cetuximab in colorectal cancer (CRC), while in thyroid cancer patients, the *BRAF* V600E mutation correlates with poor prognosis. In melanoma, *BRAF* V600E mutation occurs in 60% to 80% of the patients; anti-*BRAF* therapy is being developed and recently it showed promising results in clinical trials. The IVS14+1G>A polymorphism occurs in the dihydropyrimidine dehydrogenase (DPD) enzyme; the first and rate-limiting step in the catabolic pathway that metabolizes the drug 5-fluorouracil (5-FU). This polymorphism results in a protein with little to no activity, which can lead to severe, occasionally fatal, side effects in response to 5-FU treatment. **Methods:** The Invader Plus® chemistry consists of a limited cycle PCR followed by Cleavase®-driven allele-specific signal amplification in a homogeneous single-tube format. Invader Plus® oligonucleotides were designed to the targeted regions of interest (*BRAF* and *DPD*) and the designs then ordered through a third-party oligonucleotide manufacturer. The Invader Plus® primers and probes were designed, and then tested to optimize the initial PCR conditions and the appropriate Cleavase®-dependent fluorescent detection time using a real-time PCR platform. **Results:** For *BRAF* V600E, the reaction was optimized for the semi-quantitative detection of the mutation in DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor specimens. The assay validation showed that the sensitivity of the assay is 1% on as little as 15 ng of DNA. The *DPD* IVS14+1G>A genotyping assay has three possible outcomes: homozygous negative (no polymorphism), homozygous positive (IVS 14+1G>A present) and heterozygous. The test was developed to clearly differentiate the three possible genotypes such that fluorescence ratio from the two probes would cluster in distinct non-overlapping ranges for each genotype. **Conclusions:** The analytical laboratory validation of the Invader Plus® developed assays demonstrated 100% concordance with results from an external laboratory using a different chemistry, on 20 FFPE specimens for the *BRAF* V600E assay and on 22 peripheral blood and FFPE specimens for the *DPD* IVS14+1G>A assay.

TT23. Deparaffinization with Mineral Oil: A Simple Procedure for Extraction of High-Quality DNA from Archival Formalin-Fixed, Paraffin-Embedded Bone Marrow Trephine Biopsies

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Introduction: Extracting DNA from formalin-fixed, paraffin-embedded (FFPE) archival samples represents a challenge for molecular testing due to nucleic acid degradation with often a variable yield results. Successful PCR with DNA extracted from FFPE samples are still very low. **Methods:** We extracted DNA from 12 archival FFPE bone marrow trephine biopsy samples (from 5, 10 and >18 years of storage) by using a simple protocol based on deparaffinization with molecular biology grade mineral oil followed by DNA extraction with the Qiagen FFPE kit. Comparison of this deparaffinization method with the xylene and hemo-D protocols was investigated. The quality and quantity of extracted DNA was tested by a combination of ultraviolet (UV) spectroscopy, analysis on a bioanalyzer, and real-time PCR combined with high resolution melt (HRM) analysis of different size amplicons. **Results:** DNA extraction yields for all three deparaffinization procedures were comparable. In contrast to extraction yields, high-quality FFPE DNA was not uniform for all procedures based on the bioanalyzer and UV spectroscopy results. Higher quality of extracted DNA was obtained with deparaffinization based on the mineral oil and xylene protocols. Moreover, results obtained by deparaffinization with hemo-D protocol suggested a carryover of a byproduct with absorbance at a wavelength of 230 nm. This result was confirmed by real-time PCR and HRM. Highest quality PCR amplifiable DNA was obtained by deparaffinization with the mineral oil protocol (100% for smaller products), while more variable results were obtained for the 2 other deparaffinization procedures. **Conclusions:** Besides improvement in quality of extracted DNA, the use of mineral oil for deparaffinization has the added benefit of decreased time (20 minutes vs. 75 minutes) and a significant reduction of hands on labor (1 step vs. multiple hands-on centrifugation and decanting steps). Our results indicate that DNA extraction using mineral oil for deparaffinization is simple, fast, generates excellent quality extracted DNA and sufficient yields for retrospective studies and advocate for the adoption of this procedure in the diagnostic and research laboratories.

TT24. Pyrosequencing Is a Highly Sensitive Technique for Detection of Point Mutations in Exon 17 of *KIT* Gene Involved in Gastrointestinal Tumors, Melanoma, AML and Mastocytosis

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Introduction: *KIT* mutations have been identified in gastrointestinal stromal tumor (GIST), melanoma, acute myeloid leukemia (AML), and mastocytosis. The mutational status of *KIT* receptor tyrosine kinase has important clinical implications in targeted therapy, as a prognostic indicator and predictor of response to tyrosine kinase inhibitor therapy. The most commonly used methods for mutation identification are high resolution melting curve analysis and/or direct sequencing. We successfully identified *KIT* mutations in codons 816, 820, 822 and 823 of exon 17 using a pyrosequencing technique. **Methods:** Exon 17 *KIT* mutation analysis was performed on DNA extracted from paraffin-embedded tissue blocks including 81 GISTs, 70 melanomas, 16 mast cell disease, and on DNA extracted from banked cell pellets of 10 AML cases with inv(16) and 9 AML cases with t(8;21). The extracted DNA was subjected to PCR amplification followed by pyrosequencing analysis on the PyroMark Q24 System. Mutations involving codons 816, 820, 822 and 823 were analyzed. **Results:** Exon 17 *KIT* mutations were identified in 5 of 81 (6%) GIST cases including a N822Y mutation in 4 and a Y823H mutation in 1. One out of 70 (1%) melanoma cases demonstrated a N822Y mutation. One of 10 (10%) AML cases with inv(16), revealed a D816V mutation. Three of 9 (33%) AML cases with t(8;21) demonstrated a N822S mutation, and 9 of 16 (56%) mast cell disease cases, showed a D816V mutation. **Conclusions:** Based on our study, pyrosequencing assay is a preferred technique in reliable detection of clinically significant point mutations involving exon 17 of *KIT* gene in various types of cancer specimens. The pyrosequencing technique has a higher sensitivity, shorter turnaround time and is cost effective compared to other techniques such as high resolution melting curve and direct sequencing.

TT25. A Utility Comparison of Clinical DNA Samples Before and After Dehydration with GenTegra™ for Use on Several Common Molecular Platforms

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Introduction: Long term DNA storage has become a cumbersome, space consuming and costly venture for many clinical laboratories. With increased volumes of molecular testing, the number of archived DNA samples has risen, posing a storage space issue for many laboratories. In this study, we evaluate the feasibility of using the GenTegra™ DNA system (GenVault™), where DNA is stored in a water free environment, preventing damage from hydrolysis, oxidation and microbial growth. Previously isolated and tested clinical samples were dehydrated using the GenTegra™ DNA system and then re-hydrated and retested for the same genetic markers. **Methods:** De-identified, previously isolated and tested clinical samples were dehydrated using the GenTegra™ DNA system. Forty-five DNA samples and 5 no target controls (NTC) were used. DNA (or Tris-EDTA for the NTC) was added to the GenTegra™ DNA tube and mixed according to the manufacturer's recommendation. . The samples were dried in a desiccant bag, capped and stored at room temperature. The samples were re-hydrated to their original volume using molecular grade water, incubated at room temperature for 15 minutes, and vortexed for 1 minute. The re-hydrated DNA was then analyzed on the NanoDrop 2000 spectrophotometer and tested utilizing the AB 7500 FAST, AB 3130, Luminex and Hologic platforms. **Results:** The re-hydrated GenTegra™ DNA performed as well as original clinical samples on the various testing platforms. 10 coagulation panel, 10 Cystic Fibrosis, 10 *KRAS* and 15 fragile X samples all gave concordant results after rehydration using the respective platforms. The 260/280 ratios and DNA concentration obtained from spectrophotometer analysis were similar to those previously obtained. **Conclusions:** The GenTegra™ DNA system appears to be a viable option for storage of clinical DNA samples. Its performance on multiple platforms

indicates the integrity of the DNA was not altered during the dehydration and hydration process. The GenTegra™ DNA matrix did not interfere with these testing systems.

TT26. The Optimization of Copy Number Analysis of FFPE Tumor DNA by Array-CGH for Implementation into a Clinical Diagnostic Laboratory

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Introduction: Array comparative genomic hybridization (aCGH) has evolved as a valuable tool for the identification of genomic copy number alterations in tumors. Reliable aCGH analysis requires tumor cell enrichment of specimens; therefore the ideal specimen would have a high percentage of tumor cells. While genomic DNA (gDNA) extracted from fresh frozen tissue is of high integrity and easier to label, formalin-fixed, paraffin-embedded (FFPE) material allows for the assessment of tumor cell content within the specimen and subsequent sampling of tumor rich areas for aCGH analysis. The goal of this study was to optimize a procedure for the isolation and labeling of gDNA from tumor enriched areas of FFPE specimens for subsequent aCGH analysis. **Methods:** DNA was extracted from six pairs of matched FFPE (5x20-µm sections) and fresh frozen specimens representing a variety of lymphoma types. The six FFPE DNAs were labeled chemically using the Agilent Technologies Oligo aCGH Labeling *KIT* (ULS) or enzymatically using the Enzo Life Sciences CGH Labeling Kit (Enzo), while gDNAs extracted from the fresh frozen specimens and tumor enriched FFPE cores (1.5x2-µm cores) were labeled enzymatically. All DNAs were hybridized on a custom designed, targeted oligonucleotide array. Data from scanned arrays was extracted and analyzed using Agilent software. Agilent's "Genomic Workbench Lite" was used to visualize, detect, and analyze copy number changes. Aberrations detected in fresh or FFPE specimens were confirmed via QPCR using a candidate mapped gene. **Results:** A comparison of the quality of hybridizations for the six FFPE specimens across labeling methods, and to those obtained for matched fresh tissue revealed higher signal to noise ratios for Enzo-labeled FFPE gDNA compared to the ULS-labeled gDNA, and values comparable to those obtained for fresh frozen tissue. DLRSpread for Enzo-labeled FFPE gDNAs was substantially lower than that for ULS-labeled FFPE gDNAs, which was comparable to those obtained for fresh frozen tissue. The log ratio profiles for Enzo-labeled FFPE gDNAs exhibited overall lesser scatter than for ULS-labeled FFPE gDNAs such that aberration detection was more challenging for the latter. The isolation and labeling protocol was successfully used to identify copy number changed in gDNA isolated from tumor enriched FFPE cores using aCGH. **Conclusions:** We optimized a protocol for the isolation and labeling of FFPE gDNA from sections and tumor enriched cores for aCGH analysis. The enzymatic method of FFPE gDNA labeling was identified as the more robust method for aCGH of such technically challenging specimens.

TT27. Rapid Genotyping of *HLA-B27* by Direct Real-Time Allele-Specific PCR and Melting Curve Analysis without DNA Preparation from Whole Blood

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Introduction: *HLA-B27* genotyping is a common laboratory molecular test for several rheumatic diseases. Despite real-time melting curve analysis for *HLA-B27* genotyping is introduced, this technique is required for purified DNA as template. We develop a rapid genotyping technique using direct real-time allele-specific PCR and melting curve analysis without DNA extraction from whole blood. **Methods:** Two hundred twenty samples of randomly selected K2EDTA-treated venous blood were used in the study. Because hemoglobin is a strong quencher, we treated 300 µL of whole blood by 600 µL of rapid RBC lysis solution at room temperature for 3 min. After centrifugation and discard of supernatant, cell pellet was diluted with 200 µL of phosphate-buffer saline. A leukocyte suspension was used directly in the direct real-time allele-specific PCR using sequence-specific primers. We performed direct real-time PCR and melting curve analysis by AnyDirect qPCR Hot master mix (BioQuest, Seoul, Korea), EvaGreen fluorescence dye (Biotium, Hayward, CA), and Rotor-Gene Q (Qiagen, Valencia, CA). For confirmation of results of direct real-time melting curve analysis, we also confirmed by a commercial *HLA-B27* genotyping kit (BioWithus, Seoul, Korea) using purified DNA as template. **Results:** Melting temperatures of HLA-B27 positive melting peak and internal control melting peak were 87.4±0.3℃ and 89.5±0.4℃, respectively. Concordance rate with direct real-time melting curve analysis and commercial kit was 100%. Total running time and handling time of direct real-time melting curve analysis for 15 samples was 1.8 hr and only 15 min, respectively. **Conclusions:** Direct real-time allele-specific PCR and melting curve analysis for *HLA-B27* genotyping is a reliable, labor-saving and economic technique.

TT28. Evaluation of the Maxwell Automated Nucleic Acid Extraction Instrument Using Formalin-Fixed, Paraffin-Embedded Tissue Specimens

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Introduction: Molecular Diagnostic Laboratories are often required to isolate nucleic acid from formalin-fixed, paraffin-embedded (FFPE) tissue sections for downstream molecular testing. Extraction of high quality/quantity DNA from these samples can be challenging and labor-intensive. In general, automation in the clinical lab can lead to improved standardization and reduced hands-on and total testing time. In this study, the Maxwell Automated Nucleic Acid Extraction Instrument was evaluated and compared to a manual extraction method. **Methods:** DNA was isolated from 13 microdissected, unstained FFPE tissue specimens (2 skin, 11 colon) using a manual method (QIAamp DNA FFPE Tissue Kit, Qiagen) and an automated method (Maxwell 16 FFPE Plus LEV DNA Purification Kit, Promega) according to the manufacturer's protocol. The concentration and purity of the extracted DNA specimens was determined by spectrophotometric readings at 260 and 280 nm. Total amplifiable DNA was determined using real-time PCR amplification on the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument. All specimens were analyzed for the *BRAF* V600E mutation using PCR and pyrosequencing on the PyroMark Q24 system. The colon specimens were analyzed for microsatellite instability (MSI) using the Promega MSI Analysis System v1.2 on the Applied Biosystems 3500 Genetic Analyzer. **Results:** In general, DNA yield, as determined by 260 nm spectrophotometric readings, was approximately 40% greater using the Maxwell automated extraction method versus the Qiagen manual extraction method. The total amount of amplifiable DNA, as determined by real-time PCR, was approximately 200% greater using the Maxwell method versus the Qiagen method. DNA from both isolation methods yielded interpretable data for the MSI and *BRAF* assays using a fixed amount of input DNA. In general, there was no significant difference in MSI peak heights or *BRAF* pyrosequencing signal intensities from the samples extracted by both methods. **Conclusions:** Samples isolated by the automated Maxwell method yielded higher total quantity, and amplifiable DNA compared to samples isolated by the manual Qiagen kit, indicating that DNA specimens isolated by the Maxwell method could generate more test results from the same amount of starting tissue. The Maxwell method had a higher cost for reagents, but a significant savings in labor expenditure compared to the Qiagen method.

TT29. Implementation of Histology Sectioning in a Clinical Molecular Diagnostics Laboratory: A Process Improvement Project

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Introduction: Molecular testing of paraffin-embedded tissue specimens requires pathologist review and assessment of tumor cell content to determine specimen adequacy. Some cases may also require enrichment by slide-based microdissection techniques. These processes require histologic sectioning and staining of the specimen, techniques generally performed in a clinical histology laboratory. Our clinical Molecular Diagnostics laboratory is located off-site from the main anatomic pathology laboratory, requiring transport of specimens to and from the molecular laboratory via courier. Receipt of specimens that required sectioning and staining for tissue review meant return transport and routing of the specimen to the histology laboratory. Specific instructions on each specimen were issued based on the type of the molecular assay requested. This previous process required multiple hand-offs and led to misrouted or misplaced specimens and extended test turn-around times (TAT). A histology sectioning area within the Molecular Diagnostics lab was created to alleviate these issues. **Methods:** Root cause analysis and flow diagrams were created to determine areas of inefficiency. A plan to pilot histology processing of paraffin block specimens in the Molecular Diagnostics lab was developed. The goal of the pilot was to reduce the number of hand-offs/misrouted specimens and to improve test TAT. An area in the lab was identified for histology processing, all necessary equipment was obtained, and a senior histotechnologist from the hospital histology lab was recruited to train multiple molecular staff in sectioning and staining. **Results:** Measurement of the number of site changes (routing) and TAT was performed prior to and after implementation of the pilot. Routing of samples in the pre-pilot study required 6 site changes (hand-offs), while post-pilot routing required 2. Average pre-pilot and post-pilot test TAT was 7.47 and 4.96 days, respectively. The incidence of paraffin block re-sectioning due to incorrect processing was significantly reduced since the molecular staff were more familiar with the test-specific sectioning requirements. **Conclusions:** Implementation of histology procedures and pathologist review within the testing laboratory led to significant improvements in test TAT, specimen handling, and ultimately improved patient care and customer service.

TT30. Pyromaker: A Virtual Pyrogram Generator with Clinical and Research Applications

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Introduction: Some pyrosequencing traces can be complex and extremely difficult to interpret. **Methods:** We report a freely accessible software program, Pyromaker, which generates simulated traces for pyrosequencing results based on user inputs. The user inputs wild-type and mutant sequences, whether the mutation is heterozygous or homozygous, and the % tumor cells. Using this information, Pyromaker first calculates the percent mutant alleles and the percent wild-type alleles. Using either a default or custom dispensation order, it then calculates and graphs a simulated pyrogram. **Results:** Simulated pyrograms can be used for a variety of applications, such as assisting in the analysis of complex sequencing results where various hypothesized mutations can be tested, and the resultant virtual pyrograms can be pattern-matched with the actual pyrogram. It also can be used to quantify the relative percentage of cancer and normal cells within a tumor, and can display at each peak position the relative contribution of mutant and wild-type alleles. Pyromaker also can be used to detect homozygous cases, and to test various dispensation orders and identify suboptimal ones. We validated Pyromaker virtual pyrograms against the actual pyrograms for 7 common *KRAS* codons 12 and 13 mutations and the V600E *BRAF* mutation. We used Pyromaker to demonstrate that all single base mutations within codons 12 and 13 of *KRAS* generate unique pyrosequencing traces. Pyromaker was also used to optimize sequencing primer design by illustrating the utility of defining 1x using codon 11, prior to sequencing codons 12 and 13 that contain mutations. **Conclusions:** Pyromaker is a valuable tool for many aspects of pyrosequencing, especially as an aid in decoding complex pyrosequencing results. Pyromaker may also be useful for other applications such as methylation and teaching the fundamentals of pyrosequencing.

TT31. Preliminary Comparison of Sensitivity between Real-Time PCR and iCubate Cassette Versions of Two Molecular Assays

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Introduction: The iCubate system uses cassettes preloaded with all the reagents necessary to perform sample extraction, nucleic acid amplification, and product detection for a sample. The closed design of the cassette safeguards against possible amplicon contamination while providing a turnkey molecular assay system for the user. We report on the evaluation of two, single target iCubate system assays in comparison with two simplex real-time PCR assays for the same bacterial genomic targets to evaluate the sensitivity and burden of assay optimization for the iCubate platform. **Methods:** Two *S. aureus* genomic targets were chosen to compare both platforms: *aadD* (aminoglycoside adenyltransferase) and *nuc* (thermonuclease). The amplicon for each target was selected and the same amplicon region used for both assay types. Real-time PCR was performed using a single round with SYBR Green I detection. iCubate assays were performed with a proprietary two stage ARM-PCR method, with detection by hybridization to the in-cassette array and readout on the system iC Reader. Sensitivity by either method was determined by limiting dilution assay with a 95% LoD being determined on each system for each assay. **Results:** The iCubate system assays were determined to have better (lower) 95% LoD values than the comparable real-time PCR assays. Differential performance of the two assays on the two platforms was observed, with the iCubate and real-time systems showing generally similar performance levels for one target, and the iCubate assay being much better for the other target tested. In both cases, the complete iCubate assay gave LoD95 values below 500 cfu/assay. **Conclusions:** With equivalent levels of optimization performed, the iCubate system showed detection performance on par with, or better, than conventional real-time PCR for the same targets. In part this is likely due to the ARM-PCR method. While the real-time PCR reactions could likely have been brought to similar performance as the iCubate assay with sufficient optimization, our results demonstrate the iCubate system and ARM-PCR combine to provide a robust, simple platform with a simple assay optimization requirements.

TT32. Tracking and Controlling All Laboratory Processes with a Single Software Platform

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Introduction: Axial Biotech has developed a prognostic test for adolescent idiopathic scoliosis using 53 genetic markers and a logistic regression algorithm. Research and development of the prognostic test was completed in-house and our processes for developing a clinical assay were developed internally. Based on a large expected volume of tests, selection of a Laboratory Information Management System (LIMS) was critical to the development of the assay. Investigation into many LIMS options was frustrating as most were extremely costly and inflexible, limiting continued improvements to the assay. We report here the selection of an extremely flexible LIMS platform that we have expanded to encompass our entire process. **Methods:** We decided to select our LIMS system early in the assay development process to ensure integration of all components of the process. The UNIFlow™ platform was selected because of its extreme flexibility and adaptability and also because it was completely configurable by our personnel. The system provided the basic framework for developing a customized LIMS using a simple interface utilizing tags. Our IT specialist configured the system. Initially it was designed to manage the genotyping process. Subsequently, the system was extended to include DNA extraction and data review. As the processes evolved, we continued to use our own personnel to expand the LIMS to track samples from receipt, through a dynamic report generator and coordination with third party billing systems. Today the system includes account set up, accessioning, client interfacing, all laboratory processes, including direct interfacing with multiple instrument platforms, and report generation. Additionally, the system provides quality assurance oversight for director review. **Results:** Development of our comprehensive LIMS has been completed. We found that this platform could expand in multiple directions to meet all requirements. To date the LIMS system has been used to manage over 650 different institutions with 1100 plus physicians using the test. Expansion and improvements are made as needed, still using internal personnel. None of the internal personnel are computer programmers as the LIMS framework is easy to understand with minimal training. Installation of a complete flexible LIMS has enabled lower FTEs, thus reducing laboratory costs. **Conclusions:** The right LIMS can be configured and expanded to manage and control the entire testing process. This is made possible by selecting a LIMS platform that has the flexibility to adapt to any situation, both seen and unforeseen, and to have the power to do most of the configuration internally.

TT33. Ambient Temperature Collection and Storage of Whole Blood for DNA and RNA Preservation

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Introduction: The field of molecular medicine is progressing rapidly with new technologies utilizing patient DNA (genomic and mitochondrial) and RNA expression profiles to diagnose specific diseases, monitor disease progression and assess patient responses to medical treatment. However, RNA and DNA integrity in patient samples is highly labile post-collection when cell death removes genetically encoded mechanisms for repairing DNA and exposes cellular nucleic acids to numerous damaging enzymatic and non-enzymatic agents. Such damaging effects include nucleolytic attack, oxidative damage to nucleobases and the sugar-phosphate backbone and hydrolysis (in particular, base-catalyzed hydrolysis of RNA and depurination in DNA). The cumulative effect of the damage that accrues post-collection and during sample shipment and storage can severely disrupt genotyping and DNA sequencing efforts. In particular, transcription profiles are highly dynamic and can change rapidly during and after sample collection using current methods. **Methods:** Human blood was collected in K2-EDTA Vacutainers™ and in PAXgene Blood DNA tubes (PBD). Aliquots of blood from the K2-EDTA VacutainersTM were mixed with DNAgard Blood (DGB) stabilization formulation and stored at room temperature. Aliquots were also stored in the absence of stabilizer at room temperature (non-protected; NP) or frozen at -200C. Total RNA was extracted from unstabilized blood immediately after collection and from RNAgard Blood (RGB), NP and -800C samples at specified time points using the RiboPure Blood Kit (Ambion). RNA was extracted from PAXgene Blood RNA (PBR) samples using the PAXgene Blood RNA Kit. **Results:** Genomic DNA integrity in blood stored at room temperature in DNAgard Blood was equivalent to freezer storage in maintaining intact, high molecular weight gDNA for at least 8 months. Samples stored unprotected or in PAXgene Blood DNA resulted in partially degraded DNA. The gene expression profile of total RNA recovered from blood stored in RNAgard Blood (RGB) or -80ºC freezer for 7 days are similar in terms of the number and magnitude of genes up- or down-regulated. These results were much better than profiles from PAXgene Blood RNA-stored samples. **Conclusions:** These results demonstrate that ambient temperature-based preservation of DNA and RNA in whole blood provides a superior alternative to conventional (and problematic) methods of cold storage.

TT34. Sequencing DNA Using Semiconductor Chips Instead of Light

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Introduction: Ion Torrent has invented the first device, a new semiconductor chip, capable of directly translating chemical signals into digital information. The first application of this technology is sequencing DNA. The device leverages decades of semiconductor technology advances, and in just a few years has brought the entire design, fabrication and supply chain infrastructure of that industry, a trillion dollar

investment, to bear on the challenge of sequencing. The result is Ion semiconductor sequencing, the first commercial sequencing technology that does not use light, and as a result delivers unprecedented speed, scalability and low cost. **Methods:** Ion Torrent sequencing uses only natural (label-free) reagents and takes place in disposable semiconductor microchips that contain sensors that have been fabricated as individual electronic detectors, allowing one sequence read per sensor. **Results:** The system performance has been demonstrated by sequencing four bacterial genomes, ranging in genomes size and GC content from *Vibrio fisheri* (4.3Mb genome, 38% GC) to *Escherichia coli* K12 – (4.6Mb genome, 51%GC) and DH10b (4.7Mb genome, 51%GC) to *Rhodopseudomonas palustris* (5.5 Mb genome, 65%GC). Besides comprising the first genomes sequenced with post-light technology, the genomes are remarkable in the lack of data bias, which is as good, or better, than existing commercial platforms. **Conclusions:** Ion's technological applicability to routine human sequencing has also been demonstrated by utilizing Ion chips to sequence a human genome. We will show how the technology has scaled in just a few months from 1.2 million sensors in the first-generation Ion 314 chips to 6.1 and 11 million sensors in the second-generation Ion 316 and 318 chips respectively, all while maintaining the same 1- to 2-hour runtime. Additionally, Ion has successfully accomplished design, manufacture and sequencing using chips possessing the smaller 1-micron diameter well, enabling further increases in well density and sequencing throughput in subsequent chip designs. Because the heart of the system is a novel, disposable sensor, built and assembled using standard semiconductor fabrication methodologies, able to sequence without the need for intermediate enzymes or the constraints of having to image using light, the cost of genome sequencing will continue to fall with each successive generation of denser chips according to Moore's law.

TT35. Evaluation of the Asuragen Signature LTx Leukemia Translocation Panel

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Introduction: Chromosomal translocation results in the production of abnormally functioning fusion proteins, leading to diseases such as chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), acute promyelocytic leukemia (APL), and acute myelocytic leukemia (AML). In this study, we evaluated the performance of the Asuragen Signature LTx Leukemia Translocation Panel for the detection of twelve different fusion transcripts found in the hematologic malignancies mentioned as a first line screen for diagnosis. **Methods:** Total RNA was extracted using two different methods, manual extraction with the Ambion RNAqueous 4PCR Kit and automated extraction with the Qiagen RNA Cell Mini Kit on the EZ1 BioRobot, from fresh in-house residual patient whole blood and bone marrow samples, and frozen cell pellets from a cytogenetic laboratory. Total RNA input ranging from 400ng - 1000ng was required in each reaction. RNA was first reversed transcribed then amplified using a multiplex RT-PCR. Amplicons, including the GAPDH internal control, hybridized to target-specific capture probes bound to spectrally identified beads. Upon the addition of reporter molecules, fluorescence was detected and measured as median fluorescence intensity (MFI) by flow cytometry to determine the presence or absence of the specific fusion transcripts. **Results:** Of 73 samples tested, 38 passed the GAPDH MFI cutoff (>1000 MFI) and were available for further analysis. For the automated extraction, 0 out of 18 isolates from fresh in-house samples passed the GAPDH control, whereas 6 out of 8 isolates from cell pellets passed. With the manual extraction, 23 out of 28 fresh and 9 out of 19 cell pellet isolates also passed the internal control QC check. Twenty-two out of 38 results were concordant with either cytogenetic or GeneXpert analysis. Thirteen were false negatives and of these, six had a tumor ratio of less than 10%. The remaining seven false negative samples were isolates from cell pellets with higher tumor ratio. **Conclusions:** We determined that the Ambion RNAqueous-4PCR Kit method for RNA isolation is more suitable for use with the Signature LTx v2.0 Leukemia Translocation Panel. This assay is useful in the initial diagnosis of hematologic malignancies but is not suitable for monitoring of minimal residual disease.

TT36. Compatibility of Cellulose-Based DNA Purification Chemistries with Affymetrix Arrays

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Introduction: The quality of nucleic acid preparations can affect the outcome of microarray studies. Poor absorbance purity ratios are often used as a predictor of array performance. Typically nucleic acids used for microarray studies are purified by traditional precipitation-based or more convenient, silica-based methods. However, the carryover of alcohols and salts can affect absorbance ratios and interfere with subsequent microarrays. The use of cellulose as a binding matrix for nucleic acid purification relies on different purification chemistries, is amenable to a variety of purification formats and sample types, and is an ideal option for microarray sample preparation. **Methods:** We examined the use of new cellulose-based purification chemistries from Promega Corporation for preparing nucleic acid for microarray analysis. DNA was isolated from whole blood in triplicate by each purification method. A range of cellulose-based purification formats were examined: columns and particles, manual and automated, small (<0.5ml) and large (3-10ml) sample volume. Three new reagent systems were tested: the ReliaPrep™ Blood gDNA Miniprep Isolation System, ReliaPrep™ Large Volume HT gDNA Isolation System, and Maxwell® 16 LEV Blood DNA Kit (Promega Corporation). For comparison, samples were also prepared by more traditional techniques: silica-based QIAamp DNA Blood Mini Kit (Qiagen) and precipitation-based Wizard® Genomic DNA Purification System (Promega). All DNA were analyzed by UV absorbance and then tested for compatibility with the Affymetrix Genome-wide Human SNP Array 6.0. **Results:** All nucleic acid purification kits tested yielded DNA of a concentration greater than the minimum needed for Affymetrix Array processing (50ng/µl). For small volume blood purification (200 to 300µl), Maxwell® 16 LEV Blood DNA Kit and ReliaPrep™ Blood gDNA Miniprep Isolation System gave the highest concentrations (>100ng/µl). All kits tested yielded DNA with an A260/A280 purity ratio of 1.8-1.9. The A260/A230 purity ratios were > 1.8 for the cellulose-based and precipitation-based kit but the silica-base QIAamp DNA Blood Mini Kit tended to be lower <1.7. All samples were subsequently analyzed by microarray and passed standard requirements for array processing (QC Call Rates >97%). **Conclusions:** All manual and automated formats tested yielded DNA of high purity and concentration, as evaluated by absorbance measurements, and were successfully analyzed by Affymetrix Genome-Wide Human SNP Array 6.0. This study indicates the new cellulose-based purification chemistries offered by Promega are compatible for with Affymetrix SNP Arrays.

TT37. Paraffin Block Core Punching Is an Effective Method for Tumor Enrichment for Solid Tumor Mutational Studies

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Introduction: Tumor enrichment is a critical component of many solid tumor molecular assays and can be achieved by tissue core punch (TCP). One potential disadvantage of TCP is that selection of tumor for enrichment is based upon examination of one H&E stained section. Thus, the tumor cellularity (TC) within the TCP may be different from what is seen on the evaluated section, resulting in potential reduction in TC and alteration in molecular findings. In this study we systematically evaluate changes in TC and mutational findings within TCPs. **Methods:** 47 paraffin-embedded solid tumor samples were subjected to TCP using a 1mm punching instrument. The core was re-embedded at 90° to evaluate the TC along its length, independently performed by three pathologists. 31/47 samples were microdissected for top half (TH) and bottom half (BH); DNA extraction and mutational analysis of each half was performed based on parallel clinical testing and results compared. **Results:** Nine samples had a ≥ 30% change in the TC within the core. Of 31 specimens with molecular data, six discrepancies were observed between the clinical results and the BH. In 2 cases, this finding corresponded to reduction in TC. In one case, additional testing demonstrated low-level mutational heterogeneity. Two cases demonstrated variation in detection of silent SNPs, and one case demonstrated *EGFR* T790M only in TH. One case demonstrated a discrepancy only when a low analytic sensitivity methodology was used. With the exception of the one case with low-level mutational heterogeneity, no mutations were identified in the TCP specimens that were not reported clinically. Five specimens demonstrated <30% tumor cellularity in the top half, indicating suboptimal placement of the sampling needle. **Conclusions:** Our data suggests that TCP is an effective methodology for enrichment of solid tumors however it does have limitations. TC was reduced in the lower half of approximately 30% of cores. The reduced cellularity did not reduce mutant signal strength sufficiently to diminish the overall mutational status and no false negatives were apparent in the clinical testing. Moreover, the clinical cases routinely combine 3 TCPs in the DNA extraction, which suggests that multiple cores of a single specimen may mitigate against any potential false negative due to reduction in TC over the span of the TCPs.

TT38. A High Throughput Automation System for the Cervista® HPV HR Test

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Introduction: The Cervista® HPV HR test is an in vitro diagnostic test for the qualitative detection of DNA from 14 high risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) in cervical specimens. The current FDA approved method of performing the Cervista test includes manual processing of specimens to extract DNA

with the Genfind® DNA Extraction Kit and the set up of the Cervista reaction. This report describes a new Investigational Use Only automated platform for performing the test, the Cervista HPV HR High Throughput Automation (HTA). **Methods:** All system testing was in accordance with FDA guidance document "Assay Migration Studies for In Vitro Diagnostic Devices" (*http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm092752.pdf*). **Results:** The Cervista HTA fully automates DNA extraction, reaction setup, and results processing on a single platform. The system has a compact footprint of 15.6 square feet and provides flexible processing capabilities to perform multiple sample batch sizes. A batch size of 192 samples can be processed in an eight hour shift with fully automated results generated before the next day. For a 192 sample run, hands-on time was less than one hour including deck set up and daily maintenance. More than 90% of processing time was completely walk–away. To determine system reproducibility, a 9-member sample panel including high negatives, low positives, and moderate positives was tested across three different study sites. A comparative sample panel consisting of 288 residual clinical samples with various levels of HPV was tested at three external sites. The percent negative and positive agreement were both greater than 96% for the HTA versus the FDA–approved test. The potential for cross-contamination on the HTA system was also evaluated and no cross-contamination was observed. **Conclusions:** The Investigational Use Only HTA system, for use with the Cervista HPV HR test, is a fully automated, flexible system, designed to enable high throughput screening of cervical specimens for the presence of HPV DNA with limited hands-on time.

TT39. Analysis of *JAK2* V617F Mutational Burden in Myeloproliferative Neoplasia by Deep Sequencing

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Introduction: The spectrum of Philadelphia chromosome negative myeloproliferative neoplasms (MPN) includes polycythemia vera (PV), essential thrombocythemia (ET), and acute myelofibrosis (MF). The G>T somatic mutation in codon 617 of the *JAK2* gene resulting in a substitution of phenylalanine for the naturally occurring valine residue (V617F) has emerged as a diagnostic criterion for PV, ET, and MF. Since the natural progression of MPN involves expansion of the clonal progenitor population containing the *JAK2*V617F mutation, analysis of the mutational burden can provide important clues to prognosis. Current methods for analysis of this mutation include qualitative and quantitative PCR with allele specific probes and pyrosequencing. We describe the use of next generation sequencing for quantification of mutation burden. **Methods:** With approval by the IRB for analysis of de-identified specimens, 23 genomic DNA samples were selected. All cases were previously analyzed using Ipsogen Mutascreen qualitative/semi-quantitative assay (assay sensitivity 2%). Among them, 5 were positive at various levels. 17 were borderline positives, and one was negative. These gDNA were amplified with primers flanking exon 14 of the *JAK2* gene by PCR and subjected to NextGen sequencing using a 454 Junior and the percentage of fragments carrying the V617F mutation was determined. Up to 12 specimens were performed in the same sequencing run. Positive cases were analyzed in parallel by Sanger sequencing. **Results:** The 5 positive specimens had a V617F mutation burden from 2.3% to 78%. Of 17 cases with low level positivity with the Ipsogen test, only 1 contained this mutation, which was quantified at 0.4% by deep sequencing. Average Junior read number per sample was 8697 combining forward and reverse reads. Positive cases were confirmed by Sanger Sequencing but mutation loads below 10% by deep sequencing were below the limits of detection. **Conclusions:** Deep sequencing of exon 14 of the *JAK2* gene is a sensitive and specific approach for the quantification of the V617F mutation in patients with MPN. The sensitivity of Sanger sequencing was insufficient for the detection of low levels of mutational load and the Ipsogen Mutascreen assay gave false positive values in the borderline positive range. Deep sequencing is superior in comparison to other widely used mutation detection assays when sensitivity and accuracy are required. Simplifying procedure for deep sequencing with automation is needed for future diagnostic applications of this technology.

TT41. Automated Total Nucleic Acid Purification from FFPE Tissues Using Maxwell® 16

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Introduction: Traditional methods for the purification of nucleic acids from formalin-fixed, paraffin-embedded (FFPE) tissue samples are often labor intensive and include the use of hazardous organic reagents. In addition, carryover of formalin throughout the purification process can inhibit amplification. Thus, a consistent method using appropriate extraction and purification techniques is essential for the success of purified nucleic acids from FFPE samples in downstream applications. Here, we describe an automated method for the purification of total nucleic acid from FFPE tissue sections using the Maxwell® 16 instrument that eliminates the need for toxic reagents and with minimal hands on time. **Methods:** Pre-processing of 10 micron mouse FFPE tissue sections involved a simplified protocol with no xylene or phenol extraction required. Following pre-processing, samples were placed directly into the Maxwell® 16 cartridges, and purified total nucleic acid was ready in approximately 45 minutes. All samples were eluted in 50µl of nuclease-free water. Yield and purity of the purified total nucleic acid was analyzed by the NanoDrop 1000. RNA recovery was analyzed by quantitative RT-PCR using primers specific to mouse actin. **Results:** Total nucleic acid from a variety of mouse FFPE tissue sections was successfully purified, including liver, brain, heart, kidney, and spleen. Automated RNA purification with Maxwell®16 was compared to manual purification kits from Qiagen and Invitrogen. RNA recovery was equivalent to these commonly used manual methods as determined by quantitative RT-PCR analysis. **Conclusions:** Total nucleic acid from FFPE tissue samples was successfully purified using the Maxwell® 16 instrument. Automated nucleic acid purification decreases hands on time spent manually extracting, provides more consistent results from difficult to purify sample types, and reduces the risk of RNase contamination. The low elution volume format provides for highly concentrated nucleic acid ready for downstream applications. The method does not require the use of hazardous organic solvents, providing a safer method of extraction and purification.

TT42. Utility of Whole Genome Amplification for Assessing Copy Number Variation with High Density SNP Arrays from Formalin-Fixed, Paraffin-Embedded Tissue

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Introduction: The ability to obtain sufficient high quality DNA from archival formalin-fixed, paraffin-embedded (FFPE) tissue often limits genomic analysis for researchers and clinicians alike. Of numerous methods developed to optimize the quantity of DNA extracted from FFPE tissues, whole genome amplification (WGA) has become a robust and reliable technique for obtaining sufficient genomic material for a variety of molecular applications. Previous studies suggest that DNA obtained from FFPE samples may be used on high-density single nucleotide polymorphism (SNP) arrays to provide information on SNP genotypes, chromosome copy number (CN), and loss of heterozygosity, but spurious results occur with insufficient DNA template. **Methods:** We examined the feasibility of assessing chromosome CN variation using whole-genome amplification on DNA extracted from FFPE tissue, as well as fresh frozen (FF) tissue in OCT, and high-density Affymetrix GeneChip® 500K SNP Mapping Arrays. Genomic DNA was extracted from microdissected regions (approx 2.9 mm^2) of human tissue preserved in paraffin using the GenomePlex® Tissue Whole Genome Amplification Kit (Sigma®) and from human FF tissue using QiaAmp® DNA Mini Kit (Qiagen®). Whole-genome amplification was then performed on 1.5 µl of FF or FFPE DNA using the REPLI-g® whole-genome amplification kit (Qiagen®). Genotypes were determined using the Dynamic Model Mapping Algorithm in the Affymetrix GeneChip® Genotyping Analysis Software (GTYPE 4.0) package and CN variation was assessed with Genotyping Console™ (Affymetrix). **Results:** Acceptable genotyping call rates were obtained for all unamplified DNA samples (96.3 ± 1.5%) and wgaDNA samples (93.3 ± 1.6%) from FF tissue. Call rates were significantly lower; however, for wgaDNA samples from FFPE (67.5 ± 5.1%) ($p<0.001$). Assessment of CN variation was highly consistent between unamplified and whole-genome amplified FF samples, but was clearly discordant between amplified FF and amplified FFPE samples. **Conclusions:** These results indicate that FF tissue, even if whole-genome amplified, is useful for genome-wide SNP genotyping and determining chromosome CN variation, but large discrepancies are likely to occur when using whole-genome amplification on DNA isolated from FFPE. CN variation may be affected by uneven amplification of the genome with small quantities of suboptimal DNA template extracted from FFPE samples.

TT43. Workflow Comparison of Two FDA-Cleared Factor V Leiden Detection Systems

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Introduction: To provide an operational review of the Roche Factor V Leiden Mutation Detection Kit for use on the LightCycler Instrument 1.2 and the Hologic Invader® Factor V using Invader® Plus Chemistry. Extraction methods, equipment, and total time requirements were evaluated for a similar number of samples. Both manufacturers also have Prothrombin (Factor II) Detection Kits that have equivalent workflows to the Factor V Leiden tests. **Methods:** Manufacturer's package inserts and peer reviewed publications were used to gather information to compare the methods. Multiple factors outlining the workflow of the procedure and ease of use were evaluated. Comparison data was expressed as total run time post extraction to final result for a similar number of samples. Hands-on-time included all interaction with the samples and

instrumentation. Total walk-away time associated with each run time, including all wait time where the operator is available to perform other tasks and not be present at the instrumentation, was also captured. Equipment required for both methods was also compared including equipment needed for extraction. **Results:** For 92 reportable results the Hologic Invader® Factor V Leiden Test had 20 minutes hands on time, 90 minutes cycle time, and 10 minutes for data analysis. This allowed for 90-minute operator walk-away time that was 75% of the total run time. For 90 samples the Roche Factor V Leiden Detection Kit for the LightCycler 1.2 had 45 minutes hands-on time, 114 minutes cycle time, and 45 minutes for data analysis. This allowed for 114 minute operator walk-away time that was 59% of the total run time. **Conclusions:** Both systems provide opportunities for the operator to walk-away during the testing process with the Hologic Invader® Factor V test having the highest percentage versus total run time. The Hologic Invader® test also demonstrated the least total hands-on time and had the shortest run time to result for approximately 92 samples compared to Roche LightCycler 1.2 for 90 samples. Both systems have a similar amount of equipment required post extraction. The difference between the methods occurs with the extraction. The Roche method requires the MagNA Pure LC instrument be used for reproducible DNA preparation and PCR set-up. The Hologic method does not require a specific extraction method be followed, only that commercially available extraction kits or laboratory validated methods should provide sufficient concentration at sufficient purity per the Invader® Factor V package insert.

TT44. Performance of Whole-Genome Amplified DNA Isolated from Serum and Plasma for Estimating Copy Number Variation with High Density Single Nucleotide Polymorphism Arrays

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Introduction: Defining genetic variation associated with complex human diseases requires high-quality DNA from well-characterized patients. With the development of robust technologies for whole-genome amplification, sample repositories such as serum banks now represent a potentially valuable source of DNA for genomic studies and clinical diagnostics. We assessed the performance of whole-genome amplified (wga) DNA derived from stored serum/plasma for estimating chromosome copy number (CN) variation on high-density single nucleotide polymorphism (SNP) arrays. **Methods:** Fresh serum and plasma samples were obtained from subjects who voluntarily agreed to participate in this study and gave written informed consent. DNA was extracted from 200 µl of serum or plasma using the QIAamp® DNA Blood Mini Kit. Genomic (g) DNA was isolated from peripheral blood mononuclear cells with the Puregene® DNA Purification Kit according to the manufacturer's protocol. Whole-genome amplification was then performed on 2.5 µl of serum/plasma DNA using the REPLI-g® whole-genome amplification kit. Genotypes were determined using Affymetrix GeneChip® Genotyping Analysis Software and CN variation was assessed with Genotyping Console™. **Results:** Storage time and usage history did not affect DNA extraction or whole-genome amplification yields; however, samples that had been thawed and refrozen showed significantly lower call rates (73.9 + 7.8%) compared to samples that had never been thawed (92.0 + 3.3%) (P<0.001). Genotype call rates did not differ significantly (P=0.13) between wgaDNA from never-thawed serum/plasma (92.9 + 2.6%) and gDNA (97.5 + 0.3%) isolated from whole blood. Approximately 400,000+ genotypes were consistent between wgaDNA and gDNA; however, patterns of CN variation were highly discordant between serum/plasma wgaDNA and gDNA from the same patients. The CNV in the wgaDNA samples showed spurious regions of amplifications and deletions compared to the unamplified gDNA. These regions showed much larger areas of amplification and deletions across all the chromosomes compared to the unamplified gDNA CNV. **Conclusions:** While use of stringent quality control requirements can facilitate the collection of quality SNP genotype data from wgaDNA, our data suggest that more advanced analyses, such as CN and loss of heterozygosity assessments, may be compromised due to spurious amplification during the whole-genome amplification process.

TT45. Rapid High Throughput TaqMan SNP Genotyping Assay for *FVL*, *PT*, and *MTHFR*

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Introduction: Testing for Factor V Leiden (*FVL*) 1691G>A, Prothrombin (*PT*) 20210G>A, and 5, 10- methylene tetrahydrofolate reductase (*MTHFR*) 677C>T polymorphisms is commonly performed in clinical molecular laboratories as part of a work up for thrombophilia. A variety of testing methods are commercially available that utilize different platforms and chemistries. In this study, we compare the performance of the Hologic Invader assay for these three polymorphisms with a TaqMan laboratory developed assay. **Methods:** We utilized DNA samples from 18 patients who had been previously tested for FVL, *PT*, and/or *MTHFR* polymorphisms by the Hologic Invader technology. DNA was isolated from whole blood using the EZ1 DNA Blood Kits (Qiagen, Valencia, CA). All cases were analyzed using custom TaqMan SNP Genotyping Assays for FVL (rs6025), *PT* (rs1799963) and *MTHFR* (rs1801133) polymorphisms (Life Technologies, Carlsbad, CA). Real-time PCR was performed on the AB 7500 instrument using AB 2x fast universal master mix, 10-20 ng of genomic DNA in a total reaction volume of 10 µl with the default fast cycling conditions. A post amplification plate read was used for allelic discrimination. Known wild-type and mutant samples were tested as controls in each assay run. **Results:** Of 18 patient samples, the results were as follows: *FVL* – 10 wild type (WT), 5 heterozygous and 3 homozygous; *PT* – 13 WT, 3 heterozygous and 1 homozygous; *MTHFR* – 6 WT, 7 heterozygous and 5 homozygous. There was 100% concordance between the two assays. All control samples gave the expected results. Hands-on time for this assay was approximately 45 minutes and the time to result was approximately 3 hours versus 5 hours for the Hologic Invader assay. **Conclusions:** Our study shows that the 7500 FAST TaqMan SNP Genotyping Assay is comparable to the Hologic Invader assay in detecting and characterizing *FVL*, *PT*, and *MTHFR* polymorphisms. In addition, the TaqMan assay is easy to perform, requires less technologist time, shorter incubation and an improved time to result as compared to the Hologic Invader assay.

TT46. A Workflow Integrating High-Throughput Automated RNA and DNA Extraction from FFPE Samples and Second Generation Sequencing

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Introduction: Biorepositories around the world store vast numbers of formalin-fixed, paraffin-embedded (FFPE) samples. These samples contain a large collection of phenotypic, histological and pathological data. For genomics studies, such as biomarker discovery, targeted resequencing or gene expression profiling, this resource has been largely unutilized because sample extraction can be challenging and the quality of the extracted nucleic acids is often poor. Currently, RNA and DNA extractions from FFPE samples are commonly performed manually and involve laborious protocols that are not amenable to high throughput processing. In addition there are no standard quality control methods for downstream applications such as second generation sequencing. **Methods:** In this study, we present a fully automated RNA and DNA extraction method (Tissue Preparation System, Siemens Healthcare Diagnostics; Tarrytown,NY) for IVD and research use that allows simultaneous or separate extraction of DNA and RNA from up to 48 samples in 4h with minimum operator interaction. In contrast to most other FFPE extraction protocols this method uses an innovative deparaffinization step on the instrument that does not require incubation with Xylene or other solvents. We have successfully extracted DNA and RNA from breast and prostate cancer tissue (n=100) and the study is being extended to an additional 5 different tumor tissues including breast, prostate, lung, pancreas, bladder and cervix (n=350). Qualitative and quantitative analysis was carried out by Bioanalyzer, real-time PCR and Qubit. RNA and DNA were sequenced on a Hiseq2000 second generation sequencing instrument. **Results:** Extraction of both DNA and RNA was successful in FFPE samples from breast and prostate cancer tissue. From adjacent 10µm paraffin sections the automated system, on average, extracts longer nucleic acid fragments as judged by Bioanalyzer traces and similar amounts of RNA and DNA measured by the Qubit method, compared to a standard commercially available manual protocol. Matched fresh frozen and FFPE samples are being analyzed by second generation sequencing on a HiSeq2000. **Conclusions:** We present an integrated FFPE analysis workflow that includes standardized and fully automated nucleic acid extraction and quality controls for high-quality, high-throughput preparation of FFPE samples (96 samples/8 hr work day) for downstream analysis such as resequencing or transcriptome analysis by second generation sequencing. Our integrated workflow should significantly simplify utilization of FFPE samples for downstream genomic analyses such as second generation sequencing and as a consequence unlock a largely untapped source of information.

TT47. RNA Is Isolated in High Yield and Purity from PAXgene® Stabilized Blood and from Buffy Coat Using the Maxwell® 16 SimplyRNA Method

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Introduction: The analysis of gene expression data in research has needed a critical evaluation of easy, reproducible methods for RNA extraction. The reliability of a novel chemistry on the Maxwell 16® instrument provided a middle-throughput automated procedure to isolate RNA from whole blood or its leukocyte compartment (buffy coat) to evaluate this. **Methods:** Evaluation included reproducibility, yield, and purity using the Nanodrop, Agilent Bioanalyzer and denaturing gels. A combination of quantitative RT-PCR and quantitative PCR was used to evaluate DNA contamination of the RNA. Buffy

coat from up to 10ml blood or up to 3 PAXgene® tubes could be processed in one preparation. Following isolation of the buffy coat, or collection and washing of the PAXgene® pellet, further processing involved only addition of reagents and vortexing to mix and lyse cells. All subsequent steps were fully automated. Up to 16 separate preparations were completed in approximately 1 hour on the Maxwell 16® instrument. The procedure uses no β-mercaptoethanol, so odors are minimal. **Results:** RNA concentration from one PAXgene® tube was approximately 80ng/µl or higher, in an elution volume of 50µl. RNA from the preparations reproducibly showed A260/A280 ratios 2.0 or greater. 28S/18S RNA ratios, as analyzed by Agilent Bioanalyzer, were 1.8-2.0. RIN values were 8.0-10.0. The RNA was free of significant contamination from purification reagents and DNA as determined by several measurements. Specifically, A260/A230 ratios were over 2.0; 5S bands were visible on denaturing gels and on the Bioanalyzer trace, but no contamination by DNA was visible by these methods; and DNA contamination as determined by qPCR was minimal. The mRNA could be readily measured by RT-PCR and RNA from these preparations was also suitable for analysis in microarrays. **Conclusions:** Thus, Maxwell 16® simplyRNA provides a simple and reliable way to purify high purity RNA from PAXgene® - preserved blood and from buffy coats in good yield, suitable for various downstream applications, in a medium-throughput automated format.

TT48. Validation of Genomic DNA Extraction and Amplification from Stored Samples in RNAlater

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Introduction: RNAlater® (RNAL) is a cell storage reagent that stabilizes and protects cellular RNA from fresh tissue and cell samples. Use of this reagent eliminates the need to immediately process samples when they are received for testing. Stability of human RNA in RNAL has been well established; however, DNA integrity has not been investigated. Here we report the validation of genomic DNA extractions and DNA quality from 1-week old and 10-month old RNAL-stored leukocytes for the application of reflex DNA diagnostic testing. **Methods:** Two experiments were performed: 1) DNA extraction and amplification from fresh leukocytes were compared to 1 week old RNAL-stored leukocytes (4oC); and, 2) DNA extraction and amplification from RNAL-stored leukocytes (-80ºC for 10 months) were compared to polyclonal control DNA (Invivoscribe Technologies). Three blood pools were made from 2 blood samples. Red cells were lysed followed by low speed centrifugation and PBS wash to collect leukocytes. Samples were subdivided into 4 fractions: A, B, C and D. DNA extractions were immediately performed on fractions A and B and one week later on C and D. DNA was also extracted from six 10-month-old RNAL samples. DNA was quantified (Nanodrop) and amplification was performed targeting a Specimen control size ladder (Invivoscribe Technologies, BIOMED-2). Amplification was evaluated by analyzing on a ABI3100XL. **Results:** DNA yield was 60% higher from the 1-week old RNAL samples as compared to our immediate DNA extraction. Likewise, DNA yield was obtained from all 10-month old RNAL samples. DNA amplification as judged by amplification of control DNA ladders showed no significant difference in amplification efficiency between DNA prepared at time = 0 (samples a, b), 1-week later (samples c, d) and the 10-month samples. **Conclusions:** RNAL storage allows easy archiving of cells for later RNA based molecular testing. DNA of comparable quality and yield can be obtained from cells that have been stored in RNAL solution for up to 10 months. These results suggest that DNA from appropriately RNAL stored samples can be used for reflex molecular diagnostic testing without the need to store samples separately for RNA and DNA based testing.

TT50. Improvement in Quality Control Programs through the Addition of Peer Data Analysis with Equality Peer Review

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Introduction: Accurate evaluation of test performance with run controls is vital for delivering reliable diagnostic results and making valid clinical decisions. According to CLIA and FDA regulations, clinical laboratories are responsible for monitoring the accuracy and precision of the testing process by using control procedures that will detect immediate errors and errors occurring over time. Monitoring test performance with independent quality controls can help detect and reduce deficiencies in a laboratory's internal testing process prior to the release of laboratory test results. Changes in control lots, kit lots, and personnel can lead to variation in test performance. Peer data for the performance of the same control lot with the same test kit lot gives a more robust picture of performance. In a peer review program, individual laboratories submit test results to a centralized database to compare results with other labs running the same control lot on the same kit, allowing faster identification of significant deviations without running additional controls. SeraCare's eQuality Peer Review is a web based QC data comparison tool, providing an additional layer of control and quality assurance. **Methods:** To begin implementation of eQuality for HIV RNA, data for two users of ACCURUN® 315 HIV-1 RNA Positive Control with Roche's TaqMan® HIV-1 RNA kit were uploaded to SeraCare's eQuality Peer Review program. Data from these users were compared utilizing eQuality's reports to review differences in performance (shifts and trends) over time. **Results:** The eQuality reports mean, standard deviation, %CV and graphs with user-defined or default limits. Two users in the same laboratory had means (%CV)s of 3.5E+03 (44%) and 4.4E+03 (32%) while overall mean (%CV) was 4.8E+03 (30%). CVI could also be calculated from the data by dividing the users SD by the peer SD. One user had a CVI of 1.05 while the other user's CVI was .95. **Conclusions:** The eQuality Peer Review web based QC system allows individual laboratories to create a more robust picture of test performance across time through comparative results. Combined with the use of independent quality controls, eQuality allows clinical laboratories to track test performance and to begin troubleshooting as soon as significant deviation is observed compared to peer values, instead of relying solely on the data generated within the laboratory, ensuring greater confidence and improved regulatory compliance.

TT51. A Digital Cancer Karyogram for the 21st Century: Integral Graphical Representation of Cytogenetic, Genetic and Epigenetic Level Whole Cancer Genome Data

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Introduction: Graphical correlative representation of the vast amount of information that can accrue from just a single human genome represents a formidable challenge, yet is pivotal to the realization of the full potential of personalized medicine. Cancer adds an additional layer of complexity in that cancer genomes are grossly abnormal and continuously evolving. A graphical, intuitive user interface that enables synthesis and visualization of information can effectively facilitate communication between those that curate genome-wide data into information, *i.e.,* molecular pathologists, and those that use the curated medically actionable information, *i.e.,* clinicians. Integrative approaches such as this can also facilitate the capture of important emerging correlative patterns defining critical determinants of cancer clinical behavior, which are not immediately apparent through the analysis of individual data sets. **Methods:** Emerging software tools were co-opted and enhanced to achieve this very specific yet vital aim, *i.e.,* simple intuitive representation of the most important features of a cancer genome, as we know them to date. A core component of the digital cancer karyotype (DCK) is a coded graphical representation of cytogenetic abnormalities in cancer that was developed so as to maintain the human reference genome coordinates intact. In such a manner, gross chromosome abnormalities in cancer could be directly visually correlated with multiple other layers of genome-wide data sets without the interfering effects of omnipresent chromosome numerical and structural abnormalities in cancer. In addition representation of the karyotype in such a manner allows immediate visual correlation with RNA expression levels and copy number variation. **Results:** We demonstrate in this work co-representation of complex cytogenetic, genetic and epigenetic level genome changes in cancer. Such an approach permitted the superposition of multiple layers of genome-wide data such as genome sequence, transcriptome sequence, methylation patterns, CNV, chromatin code changes, etc., potentially permitting the display of important cancer features such as genes at translocation or inversion breakpoints, important LOH areas, regions of uniparental disomy, homozygously deleted areas, predictably functionally important point mutations, epigenetic silenced areas, etc., all in one highly correlated graphical representation. **Conclusions:** This proof of principle approach is encouraging and should catalyze further developments in this important area of genome-wide multi-layered data/information representation and normalization in cancer. Rapid advances in whole genome sequence, makes this endeavor ever more important and pressing.

TT52. Exon Capture and Targeted Nextgen Sequencing for the Detection of Mutations in Hematopoietic, Gastrointestinal, and Occular Malignancies

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Introduction: The pathologic evaluation of malignant diseases increasingly requires the analysis of genetic mutations for the assignment of proper therapy. Currently, a cadre of somatic mutations provides critical information to guide the use of molecularly targeted therapies in colorectal and non-small cell lung carcinomas. Similarly, the presence of somatic mutations may predict prognosis of hematopoietic malignancies. To facilitate the application of next generation sequencing to the multi-parameter analysis of human malignancies, we developed an exon capture strategy for targeted

sequencing. **Methods:** With IRB approval for analysis of de-identified tissues, formalin-fixed, paraffin-embedded (FFPE) and fresh specimens were selected from diagnostic archives and biorepositories of our institutions, respectively, based on diagnosis and known mutation status. Three cases of tumors associated with Lynch syndrome with known germline mutations (FFPE), 3 cases of acute myelogenous leukemia (AML) (fresh), 1 patient and 2 cell lines known to harbor the *JAK2*V617F mutation (fresh), and 1 case of uveal melanoma (fresh) were selected for analysis. DNA was extracted and 275 exons from 51 genes known to harbor mutations in these tumors were captured with NimbleGen SeqCap EZ custom beads. Captured DNA fragments were sequenced using a 454 Junior. Sequence analysis was performed using GS Mapper software. **Results:** Analysis of the 3 Lynch syndrome FFPE specimens revealed mutations in DNA mismatch repair genes identical to germline mutations detected in these patients. One AML case known to have *MLL* amplification and loss of heterozygosity of *P53* demonstrated increased copies of *MLL*, decreased copies of *P53*, and a *P53* mutation in a naturally occurring hotspot. The other two cases of AML lacked mutations that recur in AML. The uveal melanoma had a mutation in codon 209 of *GNAQ*, but lacked mutations in *GNA11*, *BAP1*, and *BRAF*. There was >96% coverage of the targeted exons and >97% of captured sequences mapped to the human genome. **Conclusions:** Targeted next generation sequencing of captured exons represents an efficient and accurate approach for the characterization of human malignancies with complex mutational profiles. This strategy can be performed on one or two 10 μm sections of FFPE tissues, indicating the utility of this approach in the normal workflow of diagnostic pathology.

TT53. Validation of Results from High-Throughput Sequencing Systems Using Orthogonal Methods

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Introduction: High-Throughput (HT) sequencing systems have the advantage of providing larger data sets that enable better interpretation and greater monitoring capability. In this presentation we will describe an HT sequencing system that uses DNA polymerase and measures protons released during deoxynucleotide triphosphate incorporation to obtain sequence information. Applications described will include the detection of multiple mutations in the *KRAS* and *EGFR* genes, and identification multiple bacterial and viral pathogens along with drug resistance markers. **Methods:** A panel DNA from ten cell lines containing mutations in *CYP2D6, KRAS* and *EGFR* were used for detection of mutations with HT system and the results were confirmed by Sanger sequencing or PCR-OLA based genotyping methods using a capillary electrophoresis instrument. Nucleic acid isolated from panels of viruses and bacteria were spiked into plasma and used for pathogen identification and drug resistant mutation detection. Real-time PCR based methods and sequencing were used to confirm results obtained by the HT sequencing system **Results:** Our results indicate that the HT sequencing system can detect multiple targets (20-40) and multiple mutations (10-20) in a single run. Comparison with traditional methods indicated 100% concordance for target identification and 99% concordance for mutation detection. Higher levels of multiplexing on the HT sequencing system are being studied. **Conclusions:** Based on our initial studies it is evident that HT Sequencing systems have the accuracy to serve the needs of a testing laboratory. The high levels of multiplexing capability will generate larger data sets with less effort and reagents

TT54. Evaluation of Pyrosequencing for the Detection of *MLH1* Promoter Hypermethylation in Colorectal and Endometrial Cancers

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Introduction: *MLH1* hypermethylation and its consequent transcriptional silencing is the cause of the microsatellite instability phenotype in 10% to 15% of sporadic colorectal cancers and 20% to 30% of endometrial cancers. In both MSI+ colorectal and endometrial cancers, absence of *MLH1* hypermethylation is associated with increased risk for HNPCC (Lynch Syndrome). **Methods:** DNA was extracted from 10 FFPE tissue sections from colorectal or endometrial tumors using the Qiagen DNeasy Tissue kit (Qiagen, 69504) from a total of 9 cases. Blood DNA or normal tissue DNA was extracted to rule out germline *MLH1* hypermethylation. DNA was then bisulfite converted using the Zymo EZ DNA Methylation-Direct kit. Treated DNA was then run on the Qiagen PyroMark Q24 pyrosequencer using the Qiagen CpG *MLH1* kit. All samples had previously been tested at a reference laboratory using methylation-specific PCR. To determine the sensitivity of this assay, we performed a dilution series experiment using 7 mixtures of methylated DNA and unmethylated genomic DNA (100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56%). **Results:** Upon evaluation of the 9 cases, we found *MLH1* hypermethylation in 5 cases (3 in colorectal cancer and 2 in endometrial cancer). The other 4 cases (3 colon and 1 uterine) were negative for *MLH1* hypermethylation. Germline inactivation of *MLH1* by promoter hypermethylation was not detected in the normal tissue from these cases. We did not have any failures indicating the smaller-sized PCR products are well amplified from fragmented FFPE DNA. We did not observe significant variation in the level of methylation across the 5 CpG sites, so we determined a cutoff of >10% methylation at all 5 CpG sites to be positive for hypermethylation. Using these criteria, all 9 cases had concordant results with methylation- specific PCR. For the sensitivity assay, we observed a high degree of correlation (r > 0.996) between predicted and observed results. The sensitivity of this assay is 6%. **Conclusions:** The PyroMark Q24 pyrosequencer assay had 100% concordance for both colorectal and endometrial cancers. This assay was approved by the New York State Department of Health as a laboratory specific assay and has now been put into clinical use.

TT55. Microdissection of Cytology Diff-Quik Smears as a Source of Cellular Material for *BRAF* Mutational Analysis in Thyroid Fine Needle Aspirates

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Introduction: Analysis of *BRAF* V600E mutation status has been demonstrated to improve the diagnostic accuracy of thyroid fine needle aspirates (FNAs). Potential methods for specimen acquisition for such analysis includes extra FNA passes, utilization of needle rinse material, and harvesting cells from prepared slides. Previous studies have demonstrated that archival cytology material can be utilized to extract high-quality nucleic acids. Here we evaluate microdissection of lesional cells directly from Diff-Quik slide preparations for utilization in diagnostic molecular pathology assays. **Methods:** Thirty-seven archival Diff-Quik slides of thyroid FNAs were included in the study. Of the 37 cases evaluated, 9 were diagnosed as papillary thyroid carcinoma (PTC), 17 were indeterminate and 11 were benign. The smears were evaluated for lesional/epithelial cell content, and areas for microdissection including clusters and single lesional cells were identified followed by glass etching on the back of the slide. After de-coverslipping, the selected areas were microdissected with a manual dissecting microscope and a pneumatic cell aspiration device. DNA was isolated from the microdissected cells and utilized for direct sequencing of exon 15 of *BRAF*. **Results:** All specimens were microdissected with a range of 4 to 40 areas measuring ~2mm^2 in diameter. The areas selected for microdissection were visually determined to consist of > 50% lesional cells. All specimens had DNA yields of >150ng after extraction, with a median yield of 258ng based on spectrophotometric analysis. All specimens were successfully evaluated by direct sequencing for *BRAF* V600E mutation. Of the 37 specimens evaluated, 5 were positive for *BRAF* V600E mutation, and all positive specimens were morphologically diagnosed as PTC for a mutation detection rate of 55.5% in PTC. None of the 17 indeterminate or benign cases demonstrated the presence of a mutation. **Conclusions:** These findings demonstrate that DNA can be successfully extracted from microdissected archival cytology material for use in clinical molecular pathology assays. De-coverslipping of cytologic smears is an acceptable method for accessing lesional cells present in archival material, and microdissection directly from Diff-Quik slides allows for enrichment of the lesional cells for subsequent molecular studies. The lack of detection of *BRAF* mutation in indeterminate specimens may be attributed to a low number of tested specimens in this study.

TT56. Validation of High Resolution Melting Curve Analysis (HRMA) Using Light Cycler 480 for Screening of *TP53* Mutations in Human Malignancies

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Introduction: *TP53* mutations have been implicated in the pathogenesis of many human malignancies. The presence of these mutations often provides prognostic information for risk stratification of disease. Most *TP53* mutations are clustered in hotspot regions located in exons 5 through 9. Conventional Sanger sequencing for detection of these mutations is both time and labor intensive. High resolution melting analysis (HRMA) is a potentially efficient alternative to screen for mutations and select specific exons for further sequencing. We validated the HRMA assay for screening for *TP53* mutations in hematological and solid tumors as a part of the routine diagnostic work-up in the clinical laboratory setting. **Methods:** The HRMA assay was established and optimized using cell lines H2122 (exon 5, p.C176[C,F]), H69-1-1 (exon 5, E171[E,*]), DLD1 (exon 7, S241[S,F]), H1437 (exon 8, R267[P,R]), SW480 (exon 8, R273H; exon 8, R273H; exon 9, P309S). The assay was further validated using bone marrow aspirate and peripheral blood samples from acute myeloid leukemia/ myelodysplastic syndrome cases and formalin-fixed, paraffin-embedded tissue (FFPET) from solid tumor cases. HRMA primers were tagged with M13 for use in subsequent

Sanger sequencing. The results of HRMA and Sanger sequencing were compared. **Results:** HRMA was successfully validated for exons 5, 7, 8 and 9 of *TP53*. Assay for exon 6 is still being optimized to exclude an unwanted intronic SNP included in the original primer design. Overall, 88.5% HRMA calls (both variant and wild type) were in concordance with Sanger sequencing (exon 5 (89.6%), exon 7 (93.3%), exon 8 (85.4%) and exon 9 (89.6%)). Of the discordant results, HRMA detected a variant in exon 5 (5/48, 10.4%), exon 7 (2/30, 6.7%), exon 8 (7/48, 14.6%) and exon 9 (5/48, 10.4%), but no mutation was detected by Sanger sequencing. Importantly, no false negative results were detected by HRMA (i.e. HRMA- and Sanger+). Additionally, the HRMA assay performed well with DNA obtained from FFPE tissue of solid tumors. **Conclusions:** HRMA is a useful clinical tool to screen for *TP53* mutation, providing a cost-effective and efficient platform with marked reduction in turnaround time. Optimal performance on FFPE tissue samples makes HRMA a particularly useful technique for solid tumors, where fresh tissue is often not available.

TT57. Exonic Deletions in Known Disease Genes Detected by aCGH: Verification of Microarray Results by Standard PCR or Quantitative Real-Time PCR

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Introduction: Array based genomic hybridization (aCGH) has quickly become the method of choice for the detection of genomic copy number variants (CNVs, microdeletions/duplications), and increasingly is also used to identify exonic deletions in known disease genes utilizing targeted oligonucleotide microarray. Still, there is consensus that these results should be verified by standard well-established molecular or cytogenetic assays (i.e. PCR or FISH). This is often effortless in cases of sizeable CNVs, but more challenging for small exonic deletions, for which FISH probes are not available or not feasible. We report our initial experience utilizing standard PCR and real-time PCR for verification of exonic intragenic deletions detected by a customized targeted 135k Nimblegen® oligonucleotide microarray. **Methods:** For small intragenic deletions (one or few exons) we decided to bridge the deletions by designing flanking PCR primers and sequence the resulting fragments to identify the deletion breakpoints (standard PCR method). For larger multi-exon deletions we chose a quantitative PCR method (Dual Taqman® probe Realtime PCR) utilizing 2 probes (one in the region of the deletion and one in a control region determined by the array). Samples were run in duplicate and 3 dilutions on an ABI® 7500 FAST RT-PCR system. Copy number was defined as $2^{-\Delta\Delta CT}$, where ΔCT is the difference in threshold cycles for the sample in question versus a control DNA. **Results:** The standard PCR method was successful in many instances (n=3) of single exon deletions, where the breakpoints did not fall into repetitive intronic sequences. The quantitative PCR method (Dual Taqman® probe Realtime PCR) was much more time and labor intensive, but did yield very reliable results and confirmed deletions in all selected cases (n=6). The simultaneous use of 2 Taqman probes allowed for compensation of differences in PCR efficiency and DNA template concentrations. The quantitative PCR method was especially effective for larger multi-exon deletions. **Conclusions:** In summary, while quite reliable and easy to set-up, there are limits in applying standard PCR for the detection of intragenic exonic deletions. The quantitative PCR method (Dual Taqman® probe Realtime PCR) was much more time and labor intensive, but did yield very reliable results and confirmed deletions in all selected cases. Still, questions remain in regard to the inability of visualizing CNVs or chromosomal rearrangements with molecular assays or to clearly define nonlinear CNVs (complex rearrangements, including multiple deletions and duplications of genomic DNA).

TT58. Excellent Correlation of *Her-2/neu* Status of Gastroesophageal Carcinomas Tested by Immunohistochemistry (IHC) and Fluorescence *in Situ* Hybridization (FISH) Using the Hofmann Guideline

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Introduction: Trastuzmab has been shown to improve survival of patients with advanced gastroesophageal carcinomas that are *Her-2/neu* positive. Literature has shown an excellent correlation of IHC results and FISH results using Dako's HercepTest. Few data are available to address validity of using Ventana Pathway *Her-2/neu* kit for testing gastroesophageal carcinomas. **Methods:** A total of twenty patients were obtained from SWMC Pathology Archive, and only 18 cases were possible for analysis (two cases had insufficient tumor cells for FISH analysis). Of the 18 samples, 9 were esophageal adenocarcinomas and 9 were gastric adenocarcinomas. *Her-2/neu* IHC testing on these cases using Ventana Pathway *Her-2/neu* kit was carried out the same way as testing on breast cancers. Her-2 stain was scored based on the published recommendation on biopsy and resection specimens (Histopathology, 2008, 52:797). Her-2 FISH testing utilized the Vysis PathVysion® *HER2* DNA probe kit and the tumors were scored for *Her-2/neu* overexpression the same way as for the breast cancers. The Her-2/CEP17 ratio is calculated for determination of amplification of *Her-2/neu* genes. **Results:** Three cases that were read as 3+ positive by IHC were all amplified by FISH tests. None of the thirteen cases that were IHC negative (five case with 0 score and 8 with 1+ score) showed *Her-2/neu* gene amplification by FISH. Two cases that were read equivocal also failed to show significant *Her-2/neu* gene amplification. Our results showed an excellent correlation between *Her-2/neu* IHC testing and *Her-2/neu* FISH testing using the Hofmann's scoring criteria. **Conclusions:** *Her-2/neu* status of gastroesophageal carcinomas could be tested immunohistochemically using Ventana Pathway system. The excellent correlation of IHC results and FISH results supports the CAP recommendation of a small validation study before implementation.

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